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ORIGINAL PAPER

# Membrane transporters and carbon metabolism implicated in chloride homeostasis differentiate salt stress responses in tolerant and sensitive *Citrus* rootstocks

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**Abstract** Salinity tolerance in *Citrus* is strongly related to leaf chloride accumulation. Both chloride homeostasis and specific genetic responses to $Cl^-$ toxicity are issues scarcely investigated in plants. To discriminate the transcriptomic network related to $Cl^-$ toxicity and salinity tolerance, we have used two $Cl^-$ salt treatments (NaCl and KCl) to perform a comparative microarray approach on two *Citrus* genotypes, the salt-sensitive Carrizo citrange, a poor $Cl^-$ excluder, and the tolerant Cleopatra mandarin, an efficient $Cl^-$ excluder. The data indicated that $Cl^-$ toxicity, rather than $Na^+$ toxicity and/or the concomitant osmotic perturbation, is the primary factor involved in the molecular responses of citrus plant leaves to salinity. A number of uncharacterized membrane transporter genes, like *NRT1-2*, were differentially regulated in the tolerant and the sensitive genotypes, suggesting its potential implication in $Cl^-$ homeostasis. Analyses of enriched functional categories showed that the tolerant rootstock induced wider stress responses in gene expression while repressing central metabolic processes such as photosynthesis and carbon utilization. These features were in agreement with phenotypic changes in the patterns of photosynthesis, transpiration, and stomatal conductance and support the concept that regulation of transpiration and its associated metabolic adjustments configure an adaptive response to salinity that reduces $Cl^-$ accumulation in the tolerant genotype.

**Keywords** Chloride transport · Salt stress · *Citrus* rootstock · Genomics · Carbon metabolism

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## Introduction

It is generally accepted that long-term detrimental effects of salinity on plants are mainly produced by sodium ($Na^+$) toxicity, as suggested by numerous studies performed during the last years in model plants (reviewed in Pardo et al. 2006). However, chloride toxicity has also been recognized to be a major constraint in horticultural production on irrigated or saline soils (reviewed in Munns and Tester 2008; White and Broadley 2001; Xu et al. 2000).

This problem that is particularly relevant in *Citrus* and other woody species like avocado, stone fruit trees, and grapevines can also disturb many important cereals and vegetables (reviewed in White and Broadley 2001). Little is known concerning the mechanisms involved in primary acquisition, subcellular distribution, and long distance transport of $Cl^-$ in plants, and knowledge dealing in particular with gene responses to chloride excess is negligible.

*Citrus* has become a model system in the study of chloride toxicity given the volume of information, mostly at the physiological level, which has been published in recent years. Chloride is thought to be the major toxic component of the salt in *Citrus* plants since under salt stress NaCl and KCl exhibited similar toxicities, while $NaNO_3$ was less harmful (Bañuls and Primo-Millo 1992). Furthermore, leaf abscission was strongly dependent upon chloride accumulation (Bañuls and Primo-Millo 1995; Bañuls et al. 1997). It is currently accepted that the physiological basis for *Citrus* tolerance to salt stress is mostly related to the plant capability to restrict $Cl^-$ transport from root to shoot, a mechanism whose efficiency is particularly dependent upon rootstock performance (Iglesias et al. 2007; Gómez-Cadenas et al. 2003; Storey and Walker 1999; Romero-Aranda et al. 1998; Moya et al. 2002; Maas 1993). In citrus, the ability to exclude $Cl^-$ appears to be the combined result of several traits since chloride tolerance has often been associated with molecular, biochemical, hormonal, physiological (reviewed in Tadeo et al. 2008; Gómez-Cadenas et al. 2003; Maas 1993), and morphological factors (Moya et al. 1999).

Based on physiological data, it is predicted that chloride accumulation in *Citrus* will regulate genes involved in $Cl^-$ membrane transporters (Tadeo et al. 2008; Roberts 2006; Moya et al. 2002; Storey and Walker 1999). Primary candidate genes for chloride transport regulation are, for instance, the recently identified cation-$Cl^-$ cotransporter (*CCC*) family (Colmenero-Flores et al. 2007) and members of the $Cl^-$ channel family (*ClC*; White and Broadley 2001). However, plasma membrane transporters involved in root uptake or long-distance transport of $Cl^-$ still remain to be identified (Negi et al. 2008; Roberts 2006). In addition, other factors different to membrane $Cl^-$ transporters might certainly influence the rate of ion accumulation under salt stress conditions. In *Citrus* rootstocks, reduction of transpiration and water uptake has been unequivocally associated with decreased leaf $Cl^-$ build-up and higher tolerance (Gómez-Cadenas et al. 2003; Moya et al. 2003; Syvertsen et al. 1989). We can easily expect that this phenomenon will impact photosynthesis and carbon assimilation activity in salt-stressed rootstocks, but it remains to be clarified whether these processes are differentially regulated at the transcriptional level and if this differential regulation is relevant to the tolerance trait. Putative targets may be genes encoding for components of the photosystem machinery and some enzymes involved in carbon assimilation pathways, as observed in other transcriptomic studies (Khelil et al. 2007; Sahi et al. 2006; Seki et al. 2002; Ozturk et al. 2002; Kawasaki et al. 2001).

In order to characterize the molecular response of plants to $Cl^-$ toxicity, we took advantage of the extensive information on the physiology of salt-tolerant and sensitive *Citrus* genotypes and the recent developments of citrus molecular and genomic tools (reviewed in Talon and Gmitter 2008) to carry out a comparative genomic approach. The gene expression analyses presented here were performed on two commercial rootstocks differing in their degree of tolerance to salinity, the sensitive hybrid Carrizo citrange (CC; *Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.), a poor $Cl^-$ excluder, and the tolerant Cleopatra mandarin (CM; *Citrus reshni* hort. ex Tan.), an efficient $Cl^-$ excluder. Based on the above considerations, microarray-based transcriptional analyses were used to test: (1) that $Cl^-$ toxicity in *Citrus* leaves, rather than $Na^+$ toxicity, is a major factor in the salinity-induced response; (2) that chloride transporters and related factors influence shoot $Cl^-$ accumulation and were primary factors in the *Citrus* response to salinity. These primary responses involving chloride homeostasis, it is argued, need to be differentiated from complex downstream changes.

## Materials and methods

### Plant material

Two-year-old seedlings of Carrizo citrange (*C. sinensis* (L.) Osb. × *P. trifoliata* (L.) Raf.) and Cleopatra mandarin (*C. reshni* Hort. ex Tan.) rootstocks were selected for uniformity and transplanted to individual pots with commercial soil medium. Seedlings were first pruned and acclimatized to greenhouse conditions for a minimum of 6 months to obtain uniform leaf flushes during spring. Treatments were carried out for 12 weeks between April and June, with potted seedlings grown in the greenhouse under natural photoperiod, maximum/minimum air temperatures of 18–16°C and 29–24°C at night and day, respectively, and relative humidity between 40% and 85%. Plants were irrigated with standard nutrient solution composed of 3 mol $m^{-3}$ $Ca(NO_3)_2$, 3 mol $m^{-3}$ $KNO_3$, 2 mol $m^{-3}$ $MgSO_4$, 2.3 mol $m^{-3}$ $H_3PO4$, 17.9 μmol $m^{-3}$ Fe-ethylenediamine dihydroxyphenyl acetic acid, and trace elements (Bañuls et al. 1997) supplemented with 3 mM KCl. A total of 12 individually potted plants per time point and treatment were used to study the physiological and molecular effects of NaCl and KCl on CC and CM rootstocks over the course of 7 weeks (for microarray studies) or 12 weeks (for physiological and real-time polymerase chain reaction

(PCR) determinations). Control plants received the above treatment, whereas salt-stressed plants were additionally supplemented with either 50 mM NaCl or 50 mM KCl. Solutions were supplied in excess to avoid salt accumulation. For ion analyses, $\psi_w$ and $\psi_s$ measurements, and gene expression profiles, leaf samples were harvested periodically. Fully expanded young leaves were used for all samplings.

### $\psi_w$ and $\psi_s$ measurements

Water ($\psi_w$) and osmotic ($\psi_s$) potentials were determined in 5 mm leaf disks using the dew-point method with a C-52 sample chamber (Wescor Inc., Logan, UT, USA) connected to a psychrometer switchbox (Ps-10) and to a dew point microvoltimeter (model HT-33T, Wescor Inc.). Measurements were determined according to manufacturer's instructions. Potential values were determined in eight to 12 leaf discs obtained from four to six leaves belonging to three different plants. Turgor pressure ($\psi_P$) values were calculated from the experimentally registered $\psi_w$ and $\psi_s$ values according to the equation $\psi_P = \psi_w - \psi_s$.

### Ion content

For $Na^+$ and $K^+$ content determinations, leaves were conveniently ground and homogenized. A 0.45-μm filtrate was obtained (at least 50 mg) through extraction with deionized water. The filtrate was analyzed with an ion chromatograph (ICS-2000, Dionex) equipped with an IonPac® CS16 (250×5 mm) column. Cations were detected by suppressed conductivity with a CSRS Ultra II-4 mm suppressor column operating at 100 mA in the recycle mode. Methanesulfonic acid (30 mM) was used as eluent in isocratic mode. Column and detector cell were adjusted at an operating temperature of 40°C. Chromeleon® 6.6 chromatography management software was used for system control, data processing, and quantification. $Cl^-$ content determinations were obtained as previously described (Colmenero-Flores et al. 2007). Measurements were obtained from five seedlings, using at least four independent extractions containing a pool of three leaves taken from three different individuals.

### RNA isolation and labeling

In order to investigate gene expression profiles, total RNA was purified from control and salt-stressed plants. A completely randomized factorial design of 2×2×4 (genotype [CC and CM] × treatment [NaCl and KCl] × time [0, 2, 5, and 7 weeks]) factors with four biological replicates per time point and treatment, consisting of a pool of five to seven leaves per plant, was followed. All samples were harvested at exactly the same time of the day to prevent interfering effects of circadian rhythms. Total RNA was prepared by the "hot phenol" method (de Vries et al. 1982), treated with RNase-free DNase-I (Invitrogen) to remove traces of genomic DNA and repurified with the E.Z.N.A. Plant Mini Kit (Omega Bio-Tek, Inc.). RNA quality was assessed by UV absorption spectrophotometry using the Nanodrop spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, Delaware, USA.) and gel electrophoresis as described by Sambrook et al. (1989). Total RNA (25 μg) was labeled as previously described (Forment et al. 2005). To avoid dye-related artifacts, individual samples were always labeled with Cy5, and a reference sample, consisting of a mixture of equal amounts of RNA from all experimental samples, was labeled with Cy3.

### Microarray hybridization

The first-generation *Citrus* cDNA microarray, obtained by the Spanish *Citrus* Functional Genomics Project containing 12672 cDNA probes and representing 6,875 putative unigenes, was used in this work (Forment et al. 2005). This microarray has proven to be very useful and efficient for comparison of the several *Citrus* species since they are genetically very close (Barrett and Rhodes 1976), and therefore, *Citrus* ortholog genes have high level of identity (Terol et al. 2007). To date, the reported *Citrus* cDNA microarray hybridization has been performed with different *Citrus* plants, including several *Citrus clementina* varieties (Forment et al. 2005; Cercós et al. 2006; Ancillo et al. 2007), and *Citrus aurantifolia* (Gandía et al. 2007). Furthermore, the microarray was constructed from many libraries obtained from several species including Cleopatra mandarin and Carrizo citrange, and the origin of the different expressed sequence tags (ESTs) was not taken into account during contig assembly and analysis (Forment et al. 2005). Microarray hybridization and washing were performed as previously described (Forment et al. 2005).

### Data analysis

A GenePix 4000B microarray scanner (Axon Instruments, Inc.) and the GenePix Pro 4.1 acquisition software were used to scan the chips according to previously described parameters (Forment et al. 2005). Raw data were imported into the software R for preprocessing, visualization, and statistical analysis. Expression ratio was computed as the ratio between the background-subtracted foreground intensities of the red and green channels. *M* value was defined as the logarithm in base 2 of the expression ratio. After microarray hybridization, signal intensity, uniformity of the expression ratio over the chip surface, and normality of *M*-

value distributions were evaluated. Only microarrays with optimal hybridization data were pre-processed and normalized for further analyses, while those that did not reach the minimum quality values were discarded. Reproducibility between replicates that was further assessed indicated that the experimental system provided consistent signals in spots corresponding to the same gene and acceptable variability between biological replicates (not shown). Twenty-seven and 25 microarrays matching these criteria were thus used for CC and CM hybridizations, respectively. These included four biological replicates for all treatments except for the 2-week NaCl samples that contained three replicates for CC and a single microarray for CM. This circumstance, however, did not reduce the strength of the analyses since maSigPro, the bioinformatic tool used for the identification of differentially expressed probes, may accept unbalanced designs provided that a sufficient number of observations is available throughout the whole experiment (Conesa et al. 2006). Data normalization was done with the R package Limma (Smyth 2005), using the Print-tip Lowess function (Yang et al. 2002). Probes showing significant differential expression during the treatment were identified using the R-package maSigPro tool, a two-step regression-based method for the analysis of time course microarray data (Conesa et al. 2006). *P* values associated to the statistical analysis of differential expression were corrected for multiple comparisons using the Benjamini and Holmmer false discovery rate procedure (Reiner et al. 2003). Differences in gene expression were considered to be significant when the *P* value was smaller than 0.05 and the *M* value $\geq$0.25.

### Functional analysis

Gene ontology (GO; Ashburner et al. 2000) term annotation of array features and function-based analysis of microarray results were carried out with Blast2GO (Conesa et al. 2005). GO terms for each of the three GO main categories (biological process, molecular function, and cellular component) were obtained from sequence similarity using the application default annotation parameters. GO annotations were completed by locally running homology searches specifically against the Tair Arabidopsis database, appending InterProScan functional results to GO annotation (Quevillon et al. 2005) and applying the second layer gene ontology augmentation strategy (Myhre et al. 2006), all three functionalities available within the Blast2GO software. GO term enrichment analysis of significant differentially expressed genes was performed with the Fisher's exact test (Blüthgen et al. 2005) also present in the application. Additionally, functional classification through Munich Information Center for Protein Sequences (MIPS; http://mips.gsf.de) comparison was utilized.

### Real-time reverse transcription-PCR

Quantitative real-time reverse transcription (qRT)-PCR was performed with a LightCycler 2.0 Instrument (Roche) equipped with LightCycler Software version 4.0 according to the procedure previously reported (Colmenero-Flores et al. 2007).

### Photosynthetic efficiency

Net photosynthetic rate, stomatal conductance, and transpiration were measured with a LCpro+ portable infrared gas analyzer (ADC Bioscientific Ltd., Hoddesdon, UK) under ambient $CO_2$ and humidity. Light was provided by a photosynthetically active radiation lamp at 1,000 μmol $m^{-2}$ $s^{-1}$ photon flux density. Air flow was set at 150 μmol $mol^{-1}$, and all measurements were performed between 8 and 11 h (A.M.). Inside the chamber, average temperature was 23.0±0.5°C, and leaf-to-air vapor pressure deficit was 1.5±0.2 kPa. After instrument stabilization, measurements were made on three mature leaves (from an intermediate position on the stem) of each of the eight replicate plants. Sampling order was changed daily to avoid any environmental variations that could affect the measurements.

### Chlorophyll fluorescence parameters

Measurements were performed between 8 and 11 h (A.M) with an OS1-FL portable fluorometer (Opti-Sciences, Tyngs-boro, MA, USA). Fv/Fm measurements were performed on undamaged tissue of attached mature leaves after 30 min of dark adaptation; nine leaves per treatment were chosen randomly. Quantum yield, ΦPSII, was measured in 36 light-adapted leaves. Sampling order was changed daily to avoid any environmental variations that could affect the measurements. All calculations were performed according to Maxwell and Johnson (2000).

## Results

### Salt treatment and physiological responses

To compare gene expression profiles during salt acclimation, the salt-sensitive rootstock Carrizo citrange and the salt-tolerant Cleopatra mandarin were watered with nutrient solution without salt excess or subjected to prolonged salt treatments with either 50 mM NaCl or 50 mM KCl. The two chloride salts were used to discriminate the effects of $Na^+$ and $Cl^-$ toxicity, and physiological perturbations, ion buildup, water balance parameters, and defoliation were monitored in fully expanded young leaves after 2, 5, 7, and 12 weeks. Figure 1a showed that the pattern of cation

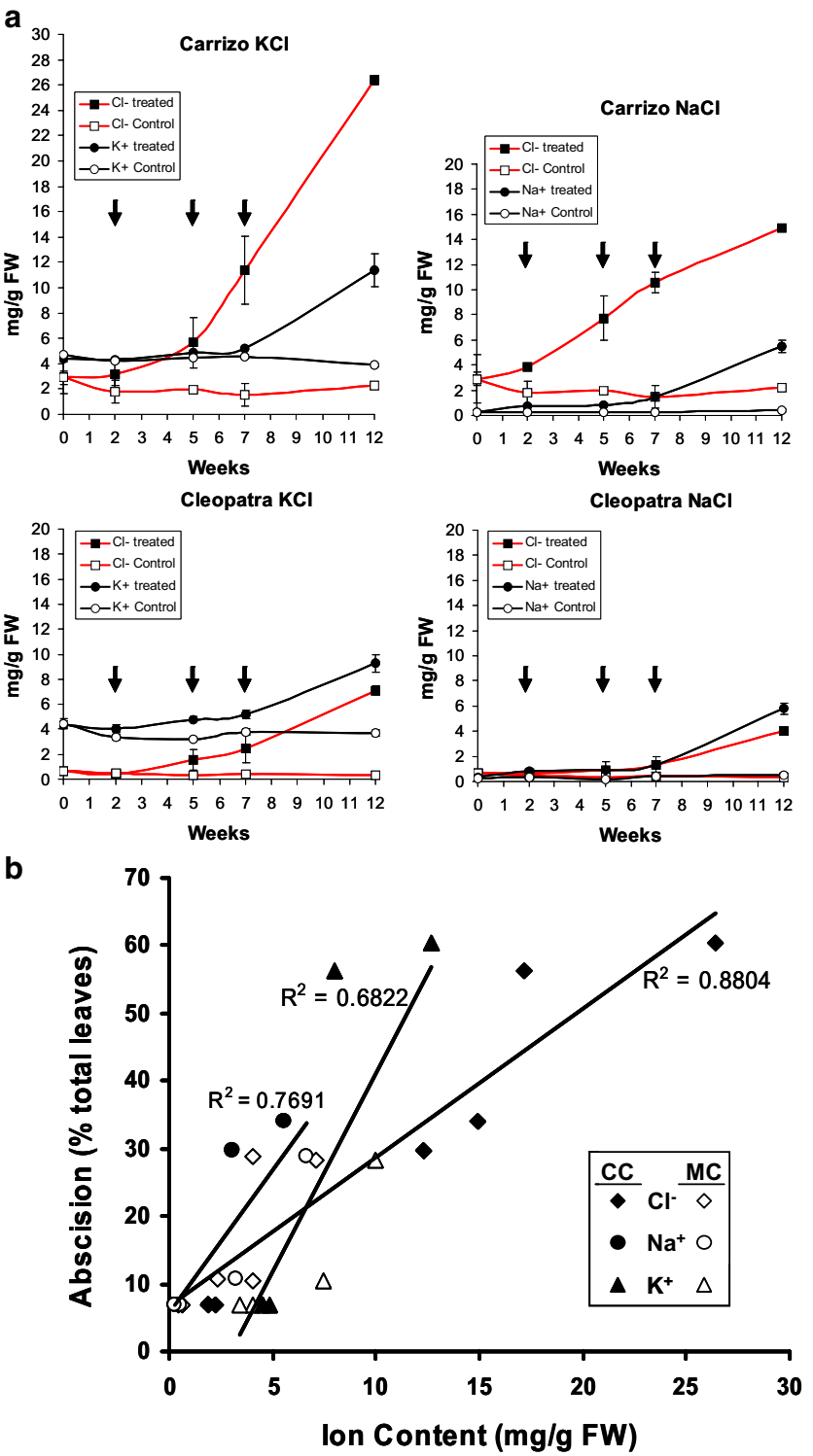



**Fig. 1** Ion ($Cl^-$, $K^+$, and $Na^+$) content accumulation (**a**) and abscission (**b**) in leaves of salt-stressed Carrizo and Cleopatra plants. Control plants received a standard nutrient solution, whereas salt-stressed plants were additionally supplemented with either 50 mM NaCl or 50 mM KCl. *Arrows* indicate harvesting times for microarray analyses. Data are means ± SD; $n \geq 4$

accumulation was similar in CC and CM leaves, while $Cl^-$ buildup was much higher in Carrizo citrange. Defoliation in both rootstocks during salt treatments correlated with high statistical significance with $Cl^-$ accumulation rather than with levels of cations (Fig. 1b). In both rootstocks, water and osmotic potentials in the salinized plants were also lower than in control plants (Supplemental Table S1) suggesting that saline ions allowed efficient osmotic adjustment enabling *Citrus* leaves to maintain similar or even to raise turgor pressure.

## Microarray analysis and qRT-PCR validation

To ensure that samples hybridized on the microarrays did not contain damaged tissue, only leaves from plants treated for 2, 5, and 7 weeks with NaCl and KCl were used for gene expression studies (Fig. 1a). After hybridization of the microarrays, only information from microarrays with optimal hybridization data were pre-processed and normalized for further analyses (see "Materials and methods"). Probes showing significant differential expression during the treatment were identified using the R-package maSigPro tool, a two-step regression-based method for the analysis of time course microarray data (Conesa et al. 2006). In order to validate the microarray data, transcript abundance of 11 probe sets (Supplemental Table S2) was quantified by qRT-PCR in leaf tissues of CC challenged with the two salts at the three time points. Quantitative data were normalized, and the resulting expression values were correlated with the microarray expression log (2) ratios. The linear regressions indicated a goodness of fit ($R^2$) of 0.92 between both kinds of analyses (Supplemental Fig. S1).

## Global gene expression

Out of the 6,875 unigenes represented in the microarray, 869 unigenes were differentially expressed in the sensitive CC rootstock, while only 208 unigenes changed in CM (Supplemental Fig. S2). Interestingly, 90% and 75% of these genes were common to NaCl and KCl in CC and CM, respectively. This observation, together with the data showing that the content of cations remained relatively low in the samples used for transcriptional analyses (Fig. 1a), clearly suggests that $Cl^-$ is the most important ion involved in the genetic response of *Citrus* plants to salinity. The variability analysis inferred for the single-gene models generated with the analysis of variance–simultaneous component analysis (ANOVA–SCA) genes methodology (Nueda et al. 2007) indicated that, in general, gene expression changed along with salinity exposure and that the major feature was the different response of CM and CC to the salt treatment, while little effect was dependent upon the kind of salt.

## Clustering and functional classification of salt-responsive genes

In Supplemental Figs. S3 and S4, clustering of significant differentially expressed genes was fitted into nine clusters according to the maSigPro analysis (Conesa et al. 2006). In CC that accumulated high $Cl^-$ with KCl and NaCl salts (Fig. 1a), 85% of the unigenes showed similar expression trends in response to both treatments (clusters 1, 2, 3, 4, 5, and 8, Supplemental Fig. S3), supporting a high correlation between leaf chloride accumulation and gene expression. Functional classification of genes was performed using the MIPS (http://www.mips.gsf.de/; see also Supplemental Fig. S5) and through Blast2GO (B2G), a research tool designed to enable gene ontology-based data mining (Conesa et al. 2005). Differentially expressed genes of CC and CM, organized according to the clustering categories, are functionally described in Supplemental Tables S3 and S4, respectively. Functional categories significantly enriched with salt-induced and -repressed genes were additionally identified through Fisher's exact tests with multiple testing correction using the B2G software (Table 1), suggesting a clear-cut different response to salinity between the tolerant and the sensitive rootstocks. The greatest differences were observed in functional categories related to stress sensing and response, photosynthesis, carbon metabolism, and energy. Subpopulations of genes that change in expression to higher levels were also analyzed. With this aim, the 25 top induced/repressed genes were classified and the most representative functional categories identified (data not shown). Functional categories of the top induced/repressed genes agreed with the enrichment analysis of GO functional categories presented in Table 1. Several of these genes were representative of the differential responses exhibited by the tolerant and the sensitive rootstocks. Thus, the mitochondrial adenosine triphosphate (ATP)-ase alpha subunit (C01009E04) and the subunit VI of the photosystem I cytochrome Cyt b6/f (Contig3077), respectively involved in ATP biosynthesis and photosynthesis, were included between the top induced genes of the sensitive CC and top repressed genes of the tolerant CM. Similarly, different chitinases, involved in defense responses, were strongly induced in CM and repressed in CC. Only two top-induced genes were common to both rootstocks, the pseudo-response regulator transcription factor (C02009B08), belonging to a phosphorelay two-component signal transduction system, and the myo-inosotol-1 phosphate synthase enzyme (Contig3351), involved in the metabolism of inositol phosphates.

## Relevant functional categories

### *Membrane transporters*

Since $Cl^-$ exclusion is an important factor determining salinity tolerance in *Citrus*, the response of $Cl^-$ transporters present in the microarray and EST collections was studied in detail (Supplemental Table S5). This functional class was not identified as an enriched category probably because most genes involved in $Cl^-$ transport mechanisms remain to be characterized. Therefore, it was also the aim of this work to identify genes potentially involved in $Cl^-$ transport. The voltage-dependent $Cl^-$ channel (ClC) is the best described family of genes encoding $Cl^-$ transporters in plants (Geelen et al. 2000; Lurin et al. 1996; Hechenberger et al. 1996).

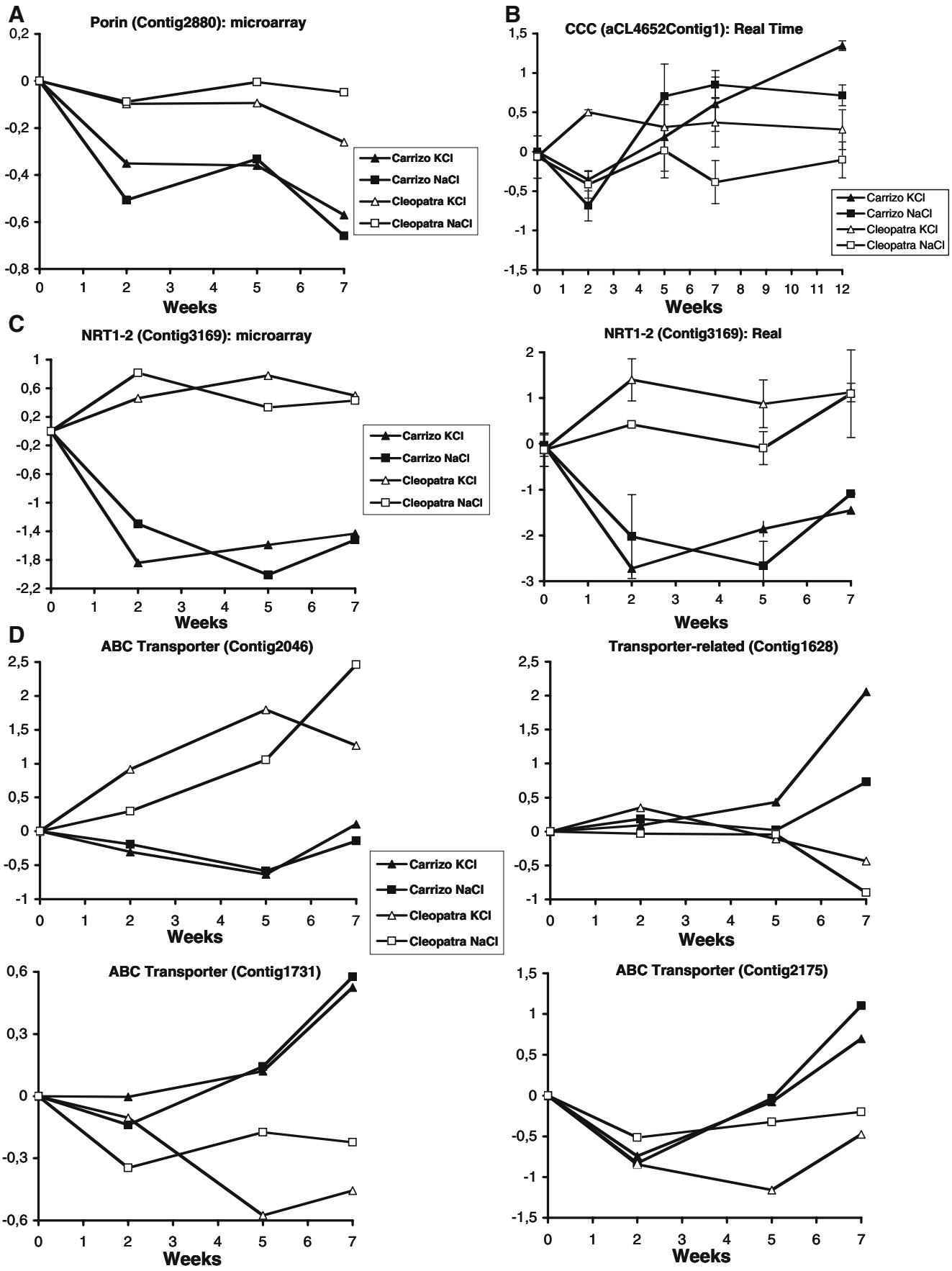
**Table 1** Enrichment analysis of GO functional categories in clusters of induced or repressed genes from sensitive Carrizo citrange and tolerant Cleopatra mandarin plants grown under salinized conditions

| GO categories | Expression | |
|---|---|---|
| | Carrizo citrange | Cleopatra mandarin |
| Stress and stimuli response | | |
| Response to abiotic stimulus | + | + |
| Response to water | + | + |
| Chromatin silencing | + | + |
| Response to stress | | + |
| Lipoxygenase activity | | + |
| Defense response to bacterium | | + |
| Response to JA stimulus | | + |
| JA biosynthetic process | | + |
| Response to ethylene | | + |
| Response to stimulus | | + |
| JA-and ethylene-dependent systemic resistance | | + |
| Defense response | | + |
| Response to ABA stimulus | | + |
| Response to wounding | | + |
| Response to external stimulus | | + |
| Chitinase activity | − | + |
| Response to hormone stimulus | − | + |
| Response to chemical stimulus | − | + |
| Response to oxidative stress | − | |
| Carbon metabolism & energy | | |
| Generation of precursor metabolites and energy | + | − |
| Thylakoid | + | − |
| Chloroplast | + | − |
| Rubisco activity | + | − |
| Carbon utilization by fixation of $CO_2$ | + | − |
| Glyoxylate metabolic process | + | |
| Chloroplast stroma | + | |
| Photosynthesis | | − |
| Photosynthetic electron transport | | − |
| Photosynthesis light reaction | | − |
| Photosynthesis dark reaction | | − |

The relative abundance of GO functional categories including differentially expressed genes is analyzed in relation to their presence in the microarray (Conesa et al. 2005). Significantly enriched functional categories are given ($P<0.005$ after Fisher's exact test for each GO term) in clusters of induced (+) or repressed (−) genes. Absence of symbol indicates that the functional category is not significantly enriched in the group of induced or repressed genes of the corresponding rootstock. Enrichment analysis of induced genes include clusters 3+4+5+9 for Carrizo citrange and clusters 1+4+5 for Cleopatra mandarin; enrichment analyses of repressed genes include clusters 1+2+8 for Carrizo citrange and clusters 2+7+9 for Cleopatra mandarin. Only pivotal categories described in the text and relevant for the discussion have been included in the table.

Members of the plant *ClC* family encode endosomal anion transporters that have primarily been involved in nitrate compartmentalization (De Angeli et al. 2006), luminal pH regulation (Fecht-Bartenbach et al. 2007) and, possibly, in vacuolar $Cl^-$ compartmentalization (Wing-Yen et al. 2006). Our results showed that the representative EST of the ClC family spotted in the 7K *Citrus* microarray, C18004G03, that corresponded to *ClC-d*, was not differentially expressed in either CC or CM in response to salinity. Furthermore, the unique bona fide chloride transporter showing differential transcript accumulation under salt stress was the outer mitochondrial membrane $Cl^-$ channel encoded by the Contig2880, a 34 KDa porin that was specifically repressed in CC (Fig. 2a). This result may be associated with a compensatory regulation of ionic homeostasis in the high $Cl^-$-accumulating rootstock. However, endosomal $Cl^-$ transporters like ClC channels or organellar porins do not appear to be directly involved in controlling $Cl^-$ exclusion mechanisms, which must operate through the regulation of root uptake or long-distance transport of this anion. Such $Cl^-$ transporters are expected to be located in the plasma membrane (PM) of the root epidermis and vascular tissues (Roberts 2006). No genes encoding PM $Cl^-$ transporters at the root periphery or the xylem parenchyma have been reported so far. The cation-$Cl^-$ cotransporter *AtCCC* gene, potentially involved in $Cl^-$ translocation at the xylem–parenchyma boundary, is probably the only exception (Colmenero-Flores et al. 2007). To quantify *CCC* gene expression in *Citrus*, we performed RT-PCR on the EST clone IC0AAA37DH06RM1, encoding for the *AtCCC Citrus* orthologue, since this gene was not represented in the 7K *Citrus* microarray. Figure 2b showed that NaCl and KCl salt treatments slightly but progressively induced the *Citrus CCC* gene in the high $Cl^-$ accumulating rootstock, indicating a possible involvement in the regulation of leaf $Cl^-$ homeostasis.

The comparative genomic approach reported here allowed the identification of uncharacterized transport protein genes differentially expressed in CC and CM that might potentially be involved in the regulation of $Cl^-$ homeostasis (see Supplemental Table S5). Interestingly, *NRT1-2* (Contig3169), a gene encoding for a member of the proton-dependent oligopeptide transporter (POT) family, was strongly repressed in CC and slightly induced in CM (Fig. 2c). This gene is homologous to putative low-affinity nitrate transporters from soybean (Yokoyama et al. 2001), and therefore, it might be involved in the regulation of anion homeostasis ($Cl^-$). *NRT1-2* expression was confirmed by RT-PCR (Fig. 2c). The ATP-binding-cassette (ABC) family protein encoded by the Contig2046, Contig1731, and Contig2175 also exhibit differential expression in the sensitive and tolerant genotypes (Fig. 2d). In animals, the *CFTR* channel family, belonging to the ABC superfamily, encodes plasma membrane $Cl^-$ channels (Jentsch et al. 2002). The S-type (slow anion channels) of guard cells shares similar gating and regulation properties with the CFTR channels. Interestingly, Contig1731 and Contig 2175


A
Porin (Contig2880): microarray
Carrizo KCl
Carrizo NaCl
Cleopatra KCl
Cleopatra NaCl
Weeks
B
CCC (aCL4652Contig1): Real Time
Carrizo KCl
Carrizo NaCl
Cleopatra KCl
Cleopatra NaCl
Weeks
C
NRT1-2 (Contig3169): microarray
Carrizo KCl
Carrizo NaCl
Cleopatra KCl
Cleopatra NaCl
Weeks
NRT1-2 (Contig3169): Real
Weeks
D
ABC Transporter (Contig2046)
Carrizo KCl
Carrizo NaCl
Cleopatra KCl
Cleopatra NaCl
Weeks
Transporter-related (Contig1628)
Weeks
ABC Transporter (Contig1731)
Weeks
ABC Transporter (Contig2175)
Weeks

◀ **Fig. 2** Expression profile of genuine and potential $Cl^-$ transporters in salt-stressed Carrizo citrange and Cleopatra mandarin rootstocks. Expression profile of the outer mitochondrial membrane $Cl^-$ channel gene obtained through microarray hybridizations (**a**). Expression profile of the *Citrus CCC* gene, determined through real-time RT-PCR (**b**). Expression profile of the *NRT1-2 Citrus* homologous gene, determined through both microarray hybridizations and real-time RT-PCR (**c**). Expression profile of other uncharacterized membrane transporters obtained through microarray hybridizations. For expression values obtained from microarrays, intensities are expressed in relation to the corresponding control value. Real-time RT-PCR values are means ($n$=4) ± SD

are homologous to ABC multidrug resistance proteins, a member of which, the *Arabidopsis* AtMRP5 protein, modulates anion channel activity in guard cells (Gaedeke et al. 2001; Suh et al. 2007). Finally, the Contig1628, homologous to sugar transporters of the major facilitator superfamily, exhibits induction in response to salinity in the sensitive genotype but repression in the tolerant one (Fig. 2d). It is also worth to mention that both cation channels and vacuolar proton pumps, membrane transporters involved in ion homeostasis, were specifically induced in the salt-sensitive CC (Supplemental Table S5).

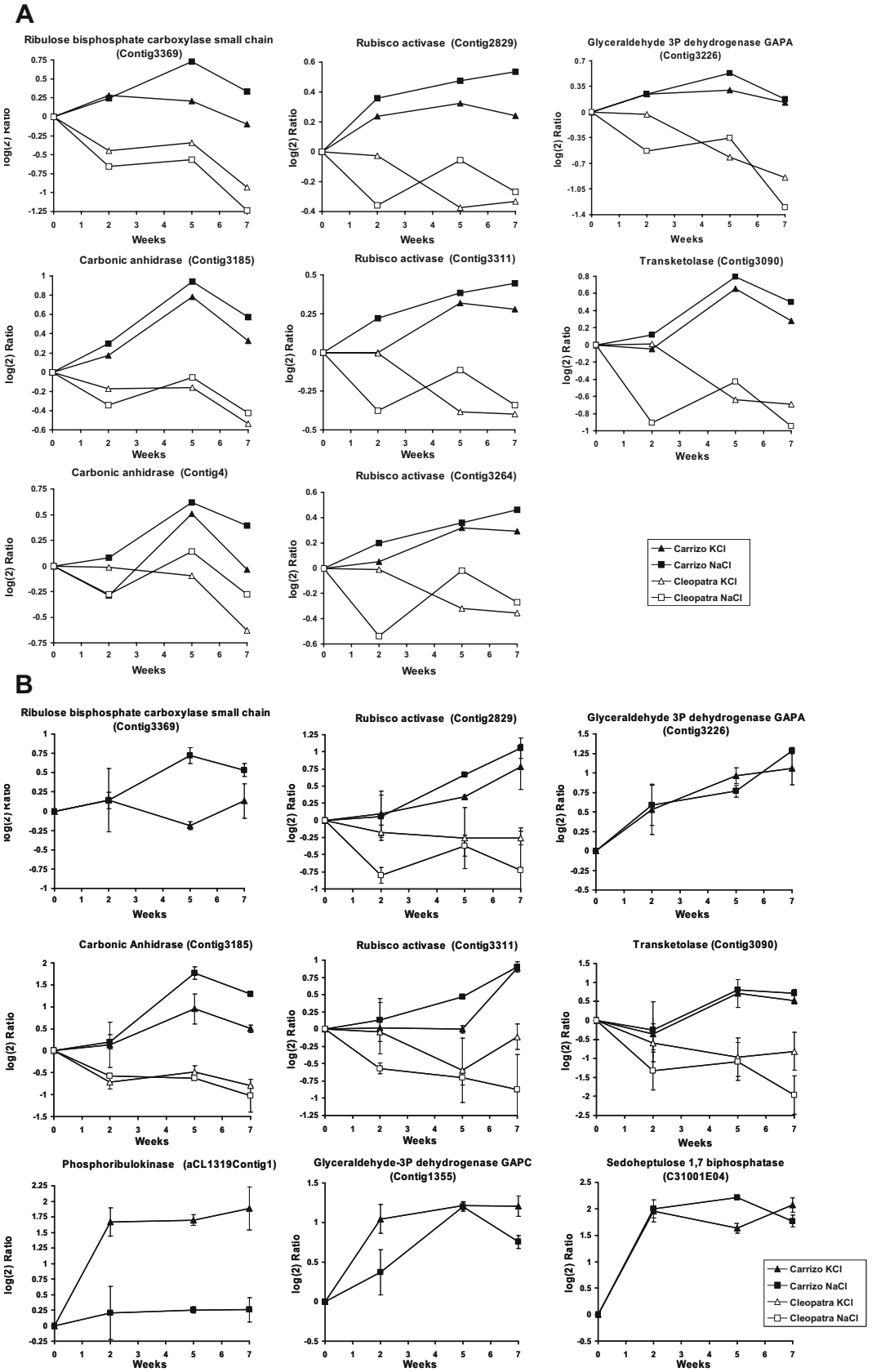
*Response to stimuli*

Analyses of functional categories showed a stronger stress response in the tolerant rootstock than in the sensitive one (Supplemental Fig. S5 and Table 1). Furthermore, for the stress-related genes, the ratio of induced vs. repressed genes was 0.82 in CC and 1.98 in CM (Supplemental Table S6). The groups termed "water stress," "salinity," "heat," and "general stress response" were mostly induced in both rootstocks, although late embryogenesis abundant (LEA) proteins were specifically repressed in CC. On the other hand, the groups "oxidative stress," "wounding response," "response to chemical stimulus," and "defense" were preferentially repressed in CC but induced in CM. The pattern of change of several gene families including peroxidases (Supplemental Fig. S6), chitinases (Supplemental Fig. S7), glutathione *S*-transferases, thioredoxins, and glutaredoxins clearly exemplified the differential stress response mechanisms triggered by both rootstocks. This may be related to differences in hormonal regulation and responses. In fact, functional categories linked to "sensing and response to hormone stimulus" were generally induced in CM and repressed in CC (Supplemental Table S7). They included mainly hormones involve in abiotic and biotic stress responses such as abscisic acid (ABA), ethylene, jasmonic acid (JA) and salicidic acid (SA). This observation suggested that the tolerant rootstock had higher capabilities to activate the stress response machinery. Moreover, biosynthetic pathways of JA and ethylene were also more repressed in CC, at least during the early 2 weeks of treatment. Lipoxygenases, involved in JA biosynthesis, were initially repressed in CC and induced in CM, although afterwards they were induced in both rootstocks, mainly in NaCl-treated plants (Supplemental Fig. S8A–G). A similar repression was observed in the JA-biosynthetic enzyme SAM:JA carboxymethyl-transferase (Supplemental Fig. S8H), whose expression pattern was further confirmed through qRT-PCR (Supplemental Fig. S8I). Genes involved in ethylene biosynthesis including ACC oxidase and ACC synthase also showed transitory repression in treated CC plants (Supplemental Fig. S9).

*Energy and carbon metabolism*

Functional categories involved in processes or cellular components related to photosynthesis and carbon assimilation were significantly induced in CC but repressed in CM (Table 1 and Supplemental Table S8), indicating that tolerant and sensitive rootstocks have developed different metabolic strategies to cope with salinity. An important set of genes encoding proteins involved in photosynthesis and carbon assimilation were preferentially induced in CC and repressed in CM (Fig. 3, Supplemental Table S8 and Supplemental Fig. S10). Differentially expressed genes included the ribulose biphosphate carboxylase (Rubisco) small chain, three Rubisco activase genes, two carbonic anhydrase genes, the phosphorylating and nonphosphorylating glyceraldehyde 3P dehydrogenase (GAPA and GAPC, respectively) genes and the transketolase gene. For some Calvin cycle enzyme genes not present in the 7K *Citrus* microarray, like the phosphoribulokinase and the sedoheptulose 1,7 biphosphatase, specific primers were designed through information obtained from *Citrus* EST databases, and transcript profiling was analyzed by qRT-PCR. The data indicated that both were induced in CC in response to salinity, although the phosphoribulokinase gene was induced in response to KCl but not in response to NaCl (Fig. 3).

In agreement with the idea that salinity stimulated the carbon and energy metabolism in CC, ATP biosynthetic processes were also differentially regulated (Supplemental Table S8). In addition, genes encoding for initial steps of glycolysis (phosphofructokinase, fructose-2P aldolase and GAPA and GAPC enzymes) were similarly up-regulated (Fig. 4a), whereas late steps of the glycolytic pathway (phosphoglycerate mutase and enolase enzyme transcripts) were preferentially repressed in CC (Fig. 4a). The repression of these genes in the sensitive rootstock, verified also by qRT-PCR (Fig. 4b), may have high physiological relevance since it indicated operation of alternative metabolic pathways different to Krebs cycle for carbon flux during salt stress (Fig. 5).


A
Ribulose bisphosphate carboxylase small chain (Contig3369)
Rubisco activase (Contig2829)
Glyceraldehyde 3P dehydrogenase GAPA (Contig3226)
Carbonic anhidrase (Contig3185)
Rubisco activase (Contig3311)
Transketolase (Contig3090)
Carbonic anhidrase (Contig4)
Rubisco activase (Contig3264)
log(2) Ratio
Weeks
Carrizo KCl
Carrizo NaCl
Cleopatra KCl
Cleopatra NaCl
B
Ribulose bisphosphate carboxylase small chain (Contig3369)
Rubisco activase (Contig2829)
Glyceraldehyde 3P dehydrogenase GAPA (Contig3226)
Carbonic Anhidrase (Contig3185)
Rubisco activase (Contig3311)
Transketolase (Contig3090)
Phosphoribulokinase (aCL1319Contig1)
Glyceraldehyde-3P dehydrogenase GAPC (Contig1355)
Sedoheptulose 1,7 biphosphatase (C31001E04)
log(2) Ratio
Weeks
Carrizo KCl
Carrizo NaCl
Cleopatra KCl
Cleopatra NaCl

◀ **Fig. 3** Expression profiles of unigenes involved in photosynthesis dark reactions and carbon assimilation in salt-stressed Carrizo citrange and Cleopatra mandarin rootstocks, determined through microarray analysis (**a**) and through real-time RT-PCR (**b**). Intensities are expressed in relation to the corresponding control value. Real-time RT-PCR values are means ($n$=4) ± SD

Finally, expression changes of several representative genes determined with qRT-PCR were also registered after 12 weeks of treatment, when stress symptoms were apparent in the sensitive rootstock (Supplemental Fig. S11). Several genes involved in Calvin cycle and carbon assimilation that were induced in CC during the initial weeks of salt stress were strongly repressed after 12 weeks. In contrast, the repression of the *NRT1-2* transporter gene was reversed during the late stages of salt treatment. As expected, expression of a typical stress gene like RD22 was more strongly induced in 12-week salinity treatments.

## Photosynthetic efficiency

The differential response to salinity of genes related to photosynthetic and carbon metabolism suggests that tolerant and sensitive rootstocks developed different metabolic strategies to cope with salinity. In order to corroborate this hypothesis, photosynthetic metabolism and other related physiological parameters were measured during salt stress in CC and CM subjected to NaCl stress. Figure 6 shows relative values in percentage between control and salt-stressed plants, while Supplemental Fig. S12 reports the absolute values, units, and variability of the data. The results indicated that salinity reduced photosynthesis and gas interchange in both kind of plants. In the tolerant rootstock, 5 weeks after the onset of the treatment and before significant foliar ion buildup occurred (Fig. 1), stomatal conductance, transpiration, performance of PSII,

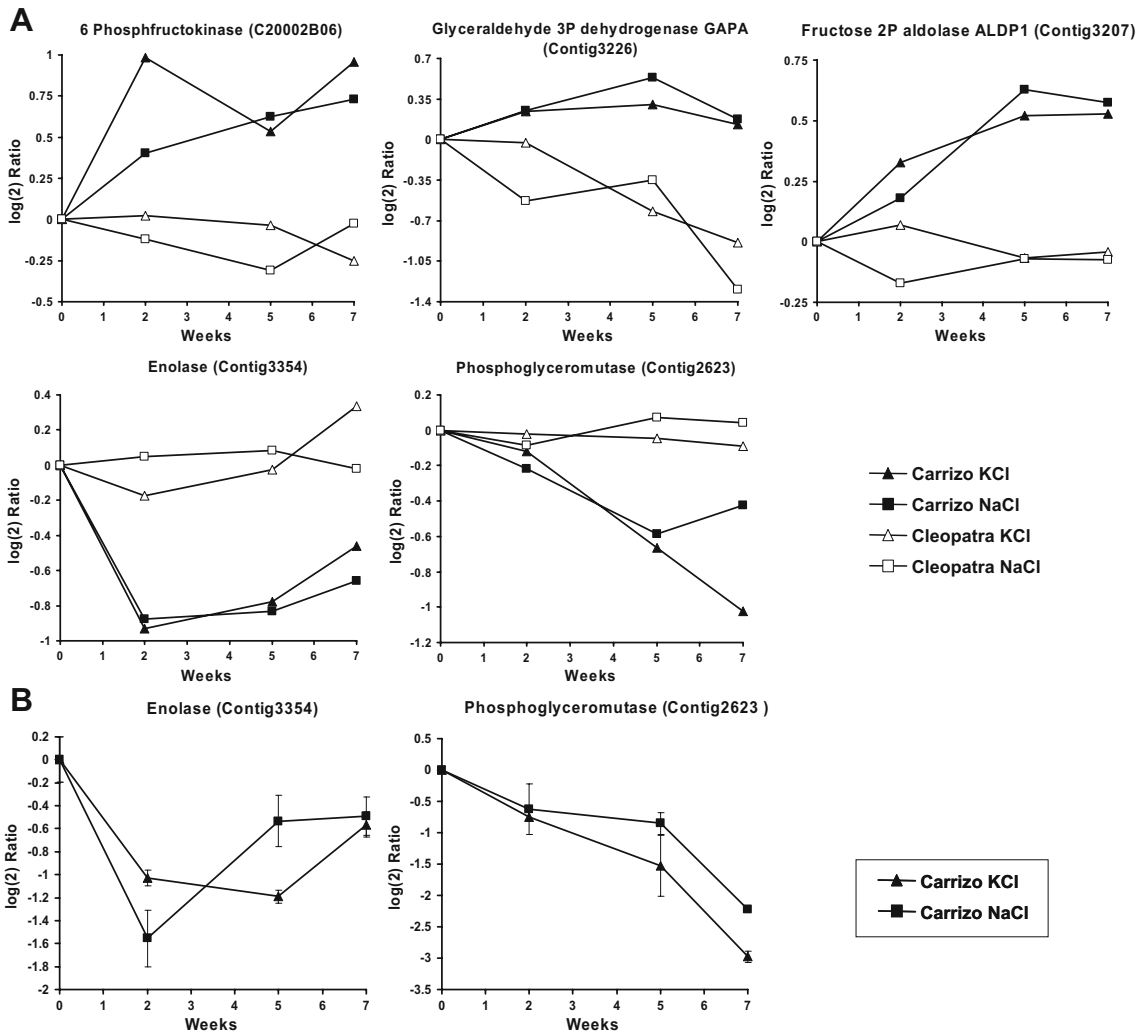



**Fig. 4** Expression profiles of unigenes involved in glycolysis in salt-stressed Carrizo citrange and Cleopatra mandarin rootstocks, determined through microarray analysis (**a**) and through real-time RT-PCR (**b**). Intensities are expressed in relation to the corresponding control value. Real-time RT-PCR values are means ($n$=4) ± SD

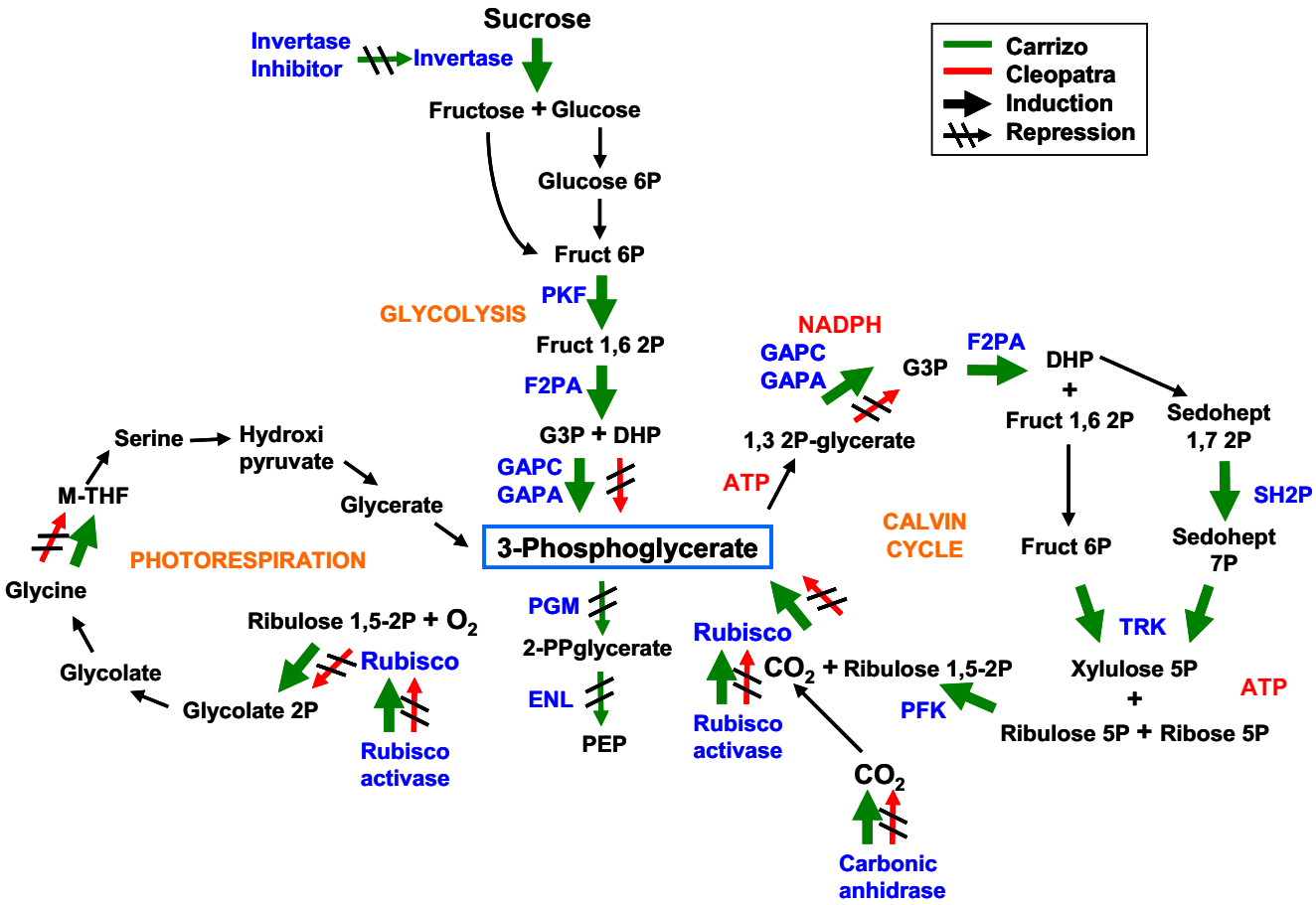



**Fig. 5** Differential metabolic flux of carbon and expression of genes involved in glycolysis, carbon assimilation, and photorespiration in salt-stressed Carrizo citrange and Cleopatra mandarin rootstocks. *ATP* adenosine triphosphate; *DHP* dihydroxyacetone phosphate; *ENL* enolase; *F2PA* fructose-biphosphate aldolase; *G3P* glyceraldehyde 3-phosphate; *GAPC* NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (nonphosphorylating); *GAPA* NADP-dependent glyceraldehyde 3-phosphate dehydrogenase (phosphorylating); *NADPH* nicotinamide adenine dinucleotide phosphate; *GDH* glycine dehydrogenase; *PGM* cofactor-independent phosphoglyceromutase; *PFK* phosphoribulokinase; *PKF* pyrophosphate-dependent phosphofructo-1-kinase; *SH2P* sedoheptulose 1,7-biphosphatase; *TRK* transketolase

and photosynthetic rate was reduced as much as 60% to 70%. In the sensitive rootstock, in contrast, inhibition of photosynthesis, stomatal conductance, and transpiration at this time was rather lower (30% to 35%) despite the considerable increase of foliar $Cl^-$ content observed in the salinized leaves. Similarly, photosynthetic machinery remained significantly more active in the high $Cl^-$ accumulator rootstock after 7 weeks of NaCl treatment. However, after 12 weeks, when intoxication symptoms were evident in sensitive Carrizo, photosynthetic efficiency and performance of PSII in this rootstock dropped to values similar or lower than those observed in the tolerant rootstock.

## Discussion

This work provides a first global microarray analysis on salinized *Citrus* plants and presents evidence supporting the notion that chloride accumulation disturbs gene expression controlling main physiological parameters. It is also revealed that salinity perception and subsequent signaling are radically different in sensitive and tolerant *Citrus*, triggering diverse responses. The regulation of efficient chloride exclusion appears to be attained through differential regulation of $Cl^-$ transporters, and the analysis identified several membrane transporters potentially implicated in chloride homeostasis such as *NRT1-2*. Processes influencing the rate of leaf ion accumulation, including transpiration or growth rates, may also have major relevance. In addition, other responses such as the transcriptional repression of light photosynthesis reactions in the tolerant CM may reduce the effects of damaging processes such as the oxidative injury that result from chloride exclusion. It was found that the compensatory induction of photosynthetic and carbon metabolism appeared to accelerate $Cl^-$ intoxication in the sensitive CC, although it might be beneficial in a different genetic

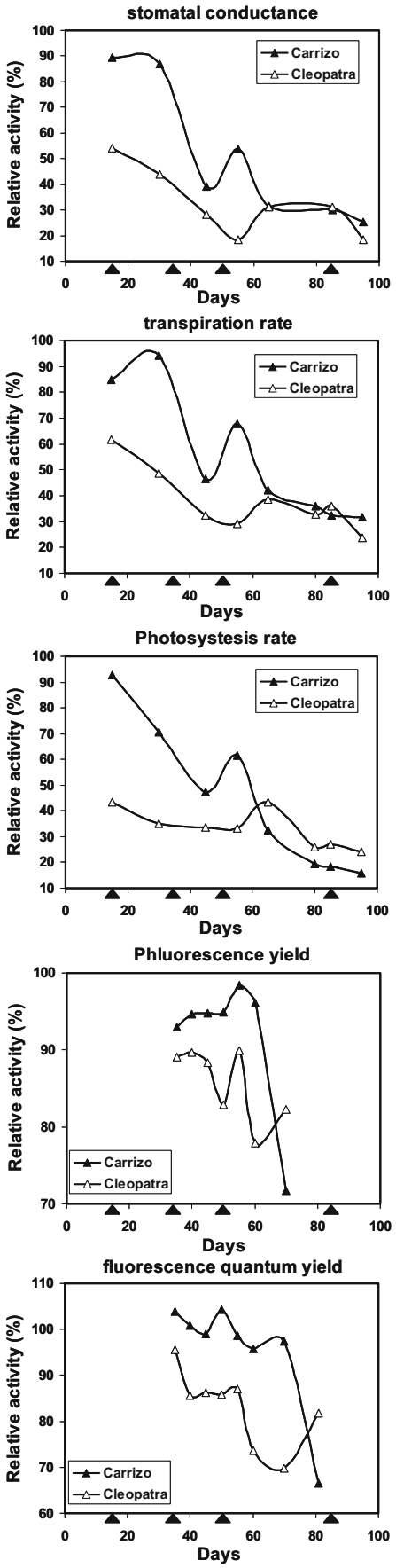



**Fig. 6** Changes in parameters associated with photosynthetic efficiency in Carrizo and Cleopatra rootstocks exposed to NaCl. Activity data of NaCl-treated plants are relative values expressed as percentage of control plants activity. *Filled triangles in the horizontal axis* indicate harvesting times used for microarrays and real-time RT-PCR analyses. Absolute values, units, magnitudes, and variability of the data are presented in Supplemental Fig. S12

background where $Cl^-$ is efficiently excluded. Many other changes shown in our analyses (see Supplemental tables) are very probably downstream from the primary response involving chloride homeostasis and are not considered in detail in the following discussion.

Most of the reports in plants dealing with NaCl stress associate long-term salt damages in leaf tissues with $Na^+$ toxicity. In order to study specific $Cl^-$ responses, in this work, the $Na^+$-dependent effect was separated from the general salinity response through the comparison of the molecular responses triggered by sodic (NaCl) vs. non-sodic (KCl) salts. The relationship between the salt-induced transcriptomic changes and the levels of $Na^+$, $K^+$, or $Cl^-$ content in leaves indicated that $Cl^-$ toxicity, rather than $Na^+$ toxicity, is the primary factor involved in the molecular responses of citrus plant leaves to salinity. These were: (1) a higher $Cl^-$ accumulation correlated with a higher number (fivefold) of differentially expressed ESTs in the sensitive CC (Supplemental Fig. S2); (2) most genes were differentially expressed, and with similar trends, in response to both NaCl and KCl treatments (Supplemental Fig. S3 and S4), suggesting that $Na^+$ is not a main salt effector of gene response in *Citrus* plants. In addition, data in Supplemental Table S1 indicated that leaf turgor pressure was maintained or even increased during salt treatments, ruling out the possibility that protoplasmic water deficit substantially contributed as a local effector to gene response in leaves. Supporting this statement, we showed that genes encoding for LEA proteins (Garay-Arroyo et al. 2000), which are typically induced in response to an osmotic stress situation that gives rise to a loss of turgor (Colmenero-Flores et al. 1999), were specifically repressed in the high $Cl^-$-accumulating rootstock CC (Supplemental Table S6), which exhibits highest leaf turgor values (Supplemental Table S1). It has been suggested, for instance, that salinity treatments rapidly produce nonsalt-specific changes in cell water relations that may transiently reduce growth rates of leaves and roots of plants (Munns 2002), but these changes take place in a short-term (minutes to hours) response (Yeo et al. 1991).

It is generally accepted that citrus tolerance to salt stress is essentially dependent upon the plant ability to restrict leaf accumulation of chloride (Romero-Aranda et al. 1998; Storey and Walker 1999; Moya et al. 2002). Direct involvement of potential $Cl^-$ transporters to salinity tolerance was analyzed through the identification of genes

encoding membrane transporters that are responsive to $Cl^-$ toxicity. A number of genes were identified that were differentially expressed not only to salt stress but also when comparing expression of the tolerant and the sensitive genotypes (Fig. 2). Among these, the Contig3169 cDNA (Fig. 2c) presents high homology to the putative soybean nitrate transporter *NRT1-2* (Yokoyama et al. 2001). They belong to the POT family, also called PTR, some of which members have been reported to catalyze the low-affinity active transport of anions like nitrate (Huang et al. 1999; Liu et al. 1999; Zhou et al. 1998), nitrite (Sugiura et al. 2007), and dicarboxylates (Jeong et al. 2004) by proton symport. Therefore, the *NRT1-2 Citrus* homolog might certainly be involved in cellular $Cl^-$ uptake through $H^+/Cl^-$ symport activity. We have recently observed that functional expression of the *Citrus NRT1-2* gene in *Saccharomyces cerevisiae* stimulates $Cl^-$ uptake into yeast cells (not shown), indicating that the approach presented here allowed the identification of novel $Cl^-$ transporters. Expression analysis of known $Cl^-$ transporters described in the literature indicated that the *Citrus CCC* gene might also play a role in chloride transport under salt conditions since accumulation of its transcript (Fig. 2b) correlated with leaf $Cl^-$ content along the experiment (Fig. 1).

Given that shoot $Cl^-$ accumulation has been also linked to transpiration (Storey and Walker 1999; Moya et al. 2003), a suggested mechanism of $Cl^-$ exclusion is the induction of stomatal closure, which reduces water uptake and absorption of soil solutes (Gómez-Cadenas et al. 2003). Provided that this mechanism appears to be exacerbated in the tolerant CM genotype, it is expected to have a strong impact in the plant physiology and metabolism and probably requires the appropriate adaptations at the molecular and biochemical levels. We showed that transcriptional regulation of genes involved in photosynthesis and carbon metabolism in the sensitive genotype CM (Figs. 3 and 4) had a clear manifestation at the physiological level (Fig. 6), suggesting the functional involvement of these responses to the salinity-tolerance trait. Since $CO_2$ is the primary sink for reducing power and energy, a significant decrease of $CO_2$ uptake in the tolerant CM may have detrimental consequences raising intracellular concentrations of ATP, NADH, and NADPH and increasing toxic levels of active oxygen species (see Tezara et al. 1999 and references therein). In this genotype, the depletion of photosynthesis light reactions through transcriptional regulation (Supplemental Table S8) can be considered a compensatory mechanism to reduce the synthesis of these intermediates and oxidative hazard. Habitually, these responses had been mainly associated with the detrimental or damaging effects of chloride intoxication (Storey and Walker 1999). However, these observations are also compatible with the assumption that chloride perception or build-up triggers transcriptional responses that regulate the homeostasis of saline ions and their metabolic adaptations, which suggests that a number of physiological alterations may be integral responses of the tolerance ability.

Interestingly, the sensitive genotype CC showed the opposite regulation and exhibited general transcriptional induction of photosynthesis, carbon metabolism, and energy (Figs. 3 and 4). This correlated also with superior photosynthesis performance, which allowed maintenance of metabolism (Fig. 6) and growth under salt stress (see also Moya et al. 2002, 2003). This might compensate for the reduced internal $CO_2$ concentration in the leaf, an observation previously reported in grapevines, another $Cl^-$-sensitive woody species (Cramer et al. 2007). An additional compensatory mechanism showed here is the transcriptional regulation of glycolysis (Fig. 4), which is expected to enhance accumulation of the intermediate 3-phosphoglycerate and feed the Calvin cycle using cellular carbohydrate stores as a source (Fig. 5). In agreement with this hypothesis, salt stress caused progressive depletion of carbohydrates (hexoses, sucrose, and starch) in *Citrus* (Arbona et al. 2005). Preservation of carbon assimilation under stress conditions allows maintenance of growth (Poorter 2002), which can be useful to dilute toxic ions in the shoot. However, it also requires a higher transpiration rate, a parameter directly linked to $Cl^-$ homeostasis in *Citrus*, which is consistent with a higher $Cl^-$ build-up in the sensitive CC. The high salinity-tolerant *Citrus* rootstock Foral-5 may combine an efficient $Cl^-$ exclusion mechanism with an active photosynthetic system at elevated saline conditions (López-Climent et al. 2008) reinforcing the concept that regulation of $Cl^-$ homeostasis is the critical factor determining NaCl tolerance in *Citrus*.

The results also revealed that the greatest differences in gene expression between the sensitive and the tolerant genotypes were also associated with stress-related processes (Supplemental Table S6), including the synthesis and action of stress-related phytohormones such as ethylene and JA (Supplemental Table S7). These observations are not surprising since it is well known that ethylene is a major inhibitor of growth and an activator of stress-protective responses (Gómez-Cadenas et al. 1998; Pierik et al. 2006) while jasmonic acid, for instance, may function as a negative hormonal regulator of transpiration (Herde et al. 1997). It is worth to mention that these changes occurred though no toxic chloride levels accumulated in the shoot of Cleopatra rootstocks (Fig. 1a), suggesting that rapid root signaling is probably involved in these responses. Genes involved in general responses to stress are expected to play a major role in $Cl^-$ tolerance although not necessarily regulating $Cl^-$ levels but for instance inducing cellular protective mechanisms and re-establishing cellular homeostasis to minimize its detrimental consequences.

The high-quality Carrizo citrange rootstock is a man-made hybrid that in principle was selected among many other citranges because the plant is vigorous, productive, and hardy. Thus, its genetic background is probably optimum for adequate development and growth in normal conditions but rather inefficient under salt-stress conditions. Cleopatra mandarins, conversely, have been selected as precious rootstocks precisely because they are rather tolerant to salinity.

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