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ORIGINAL PAPER

Tonoplast aquaporin *aqua1* over-expression modifies poplar (*Populus alba* L. clone 'Villafranca') response to salinity

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Abstract

Salinity is one of the main abiotic stresses for plant survival. In this work, response to salinity was investigated in *Populus alba* L. clone 'Villafranca' transgenic plants over-expressing *aqua1*, an aquaporin of *Populus* × *canadensis* Moench clone 'I-214' (GeneBank: GQ918138) and compared to wild-type plants (WT). Two lines with a different level of over-expression were selected. Plants were grown under control conditions (0 mM NaCl) and salinity (100 mM NaCl) for 27 days. At the end of the experiment, transgenic and WT plants were phenotypically indistinguishable. Shoot elongation and total leaf area were reduced equally in all treated plants, even if the relative reduction was lower in transgenic Line 16. The other morphological and physiological parameters measured did not evidence a response correlated to *aqua1* over-expression. Interestingly, we observed a decrease in Na concentration in the treated roots of Line 16 compared to WT. Higher polyphenols content in roots and leaves of transgenic Line 16 under control condition was observed. suggesting that *aqua1* over-expression has contributed to improve ion regulation and oxidative stress mitigation. These findings revealed that the *aqua1* over-expression helps maintain osmotic balance and efficient water transport, contributing to the stability of polyphenol profiles under salinity stress, thereby enhancing tolerance of transgenic lines. Also, our results suggest *aqua1* involvement in cell and plant growth and a possible buffering effect of *aqua1* over-expression by transcriptional and post-translational modifications that must be further investigated.

Key message

The role of *aqua1*, a poplar tonoplast aquaporin, was evaluated in *Populus alba* L. clone 'Villafranca' under salinity. *Aqua1* over-expression enhances tolerance to salinity stress limiting Na uptake in root and increasing K and polyphenols concentrations.

Keywords Poplar · Salt stress · *aqua1* · Polyphenols · Mineral elements · TIP

Introduction

Salinity emerges as one of the most relevant abiotic stressors, affecting over 1 Bha of global land area, with 30/50 Mha affected in Europe alone (Daliakopoulos et al. 2016). If the soil salinization issue is not addressed, it is projected that 50% of arable lands will be salinized by 2050 (Kibria and Hoque 2019). The effects of salinity on plant is characterized by a rapid "osmotic phase", followed by a slower "ionic phase" where salt accumulation, primarily sodium (Na), causes damage (Munns and Tester 2008) due to ion/nutrient imbalances, metabolic disorders and oxidative stress (Kibria and Hoque 2019).

The genus *Populus*, belonging to the Salicaceae family, consist of approximately 30 species distributed worldwide,

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particularly in the Northern Hemisphere (Taylor 2002). Poplars are fast-growing woody perennial plants used: (1) to produce wood-based products, such as timber, pulp and paper; (2) as a bio-energy source; (3) for the re-integration and renewal of degraded landscapes. In addition, these plants play a crucial role in carbon sequestration, aiding in mitigating Earth's climate issues (Isebrands and Richardson 2014; Todaro et al. 2017). Poplars have a small genome size, relatively to other plant species, which is four times larger than the genome of *Arabidopsis thaliana* and 40–50 times smaller than the genome of pine (Wullschleger et al. 2002). In addition, poplar can be cultivated in vitro and subjected to genetic modification using biotechnological tools. Moreover, clonal propagation ensures repeatable experiments making the genus *Populus* a group of model plants in woody tree research (Taylor 2002). The sequencing of *Populus trichocarpa* (Torr. & Gray) genome and the subsequent genomic and molecular biology advancements have further solidified its status as the "*Arabidopsis* of tree species" (Taylor 2002; Song et al. 2006; Tuskan et al. 2006; Jansson and Douglas 2007).

Populus species exhibit variability in salinity responses, with differences even observed among different clones or ecotypes of the same species. *Populus alba* L., known as white poplar, has a wide distribution area ranging from Central and Southern Europe, North Africa and Asia to the Middle east (FAO 1980) and it has been tested in salinity trials (Sixto et al. 2005; Chen and Polle 2010).

Biotechnologies offer the potential for enhancing stress tolerance in plants. This objective can be pursued applying strategies such as the over-expression of specific transcription factors, dehydrin proteins, osmoprotectants and water channel proteins (i.e. aquaporins) (Altman 2003). Aquaporins are transmembrane water channels involved in water movement across cell membranes. They are members of the Major Intrinsic Protein (MIP) family, widely spread in almost all living organisms. Aquaporins are proteins of around 30 kDa and consist of 6 transmembrane domains connected by five loops. The primary structure contains the Asn-Pro-Ala (NPA) conserved motifs and the aromatic/arginine (ar/R) selectivity filters. These proteins are usually organized as homo- or hetero-tetramers and each monomer forms an independent channel (Gomes et al. 2009). Aquaporins have been studied in several species and these channels are not only involved in water transport but also in the movement of other substrates of high physiological interest, such as hydrogen peroxide, silicon, and urea (Li et al. 2014). Plant species possess a great diversity of aquaporins, which have been divided into five main sub-families: Plasma membrane Intrinsic Proteins (PIPs), Tonoplast Intrinsic Proteins (TIPs), Nodulin26-like Intrinsic Proteins (NIPs), Small basic Intrinsic Proteins (SIPs) and uncategorized (X) Intrinsic Proteins (XIPs) (Maurel et al. 2015). In *Populus trichocarpa*, 55 genes codifying for aquaporins have been discovered (Gupta and Sankararamakrishnan 2009). Many fundamental physiological processes in plants involve aquaporins, including cell elongation, transpiration, phloem loading and unloading, and abiotic stress responses (Hu et al. 2012). Even if, in some cases, studies on single aquaporins have shown discordant results, and this is certainly due to the complex network of interactions and regulatory levels, several studies have assessed that the natural or induced over-expression of certain aquaporins could increase salt stress tolerance in different species (Sade et al. 2010; Gao et al. 2010; Hu et al. 2012; Xin et al. 2014).

In poplar, Ariani et al. (2016) produced two transgenic lines of *Populus alba* over-expressing the gene *aqua1* (GenBank: GQ918138) described by Baccio et al. (2011) and 015(2016). Moreover, a deeper characterization (Ariani et al. 2019), showed that *aqua1* cDNA encodes a 257 amino acid protein from a 771 bp nucleotide sequence. This gene shares 99% identity with a putative tonoplast intrinsic protein (TIP) from *Populus trichocarpa* (RefSeqID: XM_002331442.1). The closest blast hit identifies *aqua1* as a member of the TIP subfamily, a well-known group of aquaporins predominantly localized to the tonoplast and commonly used as a vacuolar marker in subcellular localization studies. To experimentally understand the localization, Ariani et al. (2019) co-expressed 35 S: AQUA1-GFP with RFP: AtSYP122, a plasmatic membrane marker. The confocal imaging revealed that the 35 S: AQUA1-GFP fluorescence was found to be clearly confined to internal membranes and did not overlap with the plasma membrane, further supporting the idea that aqua1 is most likely localized in the tonoplast.

Since previous studies mainly focused on aquaporins in response to water stress conditions, their role in salinity tolerance remains understudied, particularly in *Populus* species (Almeida-Rodriguez et al. 2010; Secchi and Zwieniecki 2010; Calvo-Polanco et al. 2019). For all these reasons, in this study we investigate the role of a poplar TIP under salinity using the two transgenic lines over-expressing *aqua1* in comparison with the *Populus alba* wild type.

Materials and methods

Plant growth, experimental design and treatments

Populus alba L. clone 'Villafranca' plants were grown at the Institute of Crop Science in Pisa – Italy (43°43'46.3"N 10°25'23.3"E). Wild-type plants (WT) and two transgenic lines (Line 16 and Line 24) plants over-expressing the tonoplast intrinsic protein (TIP) *aqua1*of *Populus* × *canadensis*

Moench clone 'I-214' (GeneBank: GQ918138) obtained by Ariani et al. (2016) were used. These two transgenic lines were selected since they showed a different level of *aqua1* over-expression as described in Ariani et al. (2016). After 5 weeks, plantlets from in vitro culture were transferred from vessels containing solid half-strength Woody Plant Medium (WPM; Lloyd and McCown 1980) to pots filled with perlite and closed in plexiglass boxes to maintain 100% humidity. Plants derived from in vitro culture were acclimatized in vivo for six weeks in a growth chamber under controlled environmental conditions (23:18 °C day: night temperature, 65–70% relative humidity, and 16 h photoperiod at photosynthetic photon flux density of 400 µmol m^{-2} s^{-1} supplied by fluorescent lights). During the acclimation process, the nutrient solution was gradually changed from half-strength liquid WPM to a modified Hoagland's solution at pH 6.5 (Arnon and Hoagland 1940), while relative humidity was reduced from 100% to the growth chamber humidity level (65–70% relative humidity). The modified Hoagland's solution contained: 4.00 mM of $Ca(NO_3)_2 \times 4H_2O$, 2.10 mM of $MgSO_4 \times 7H_2O$, 4.00 mM of KNO_3, 1.00 mM of $K_2HPO_4 \times 3H_2O$, 1.50 mM of NH_4NO_3, 43.67 µM of H_3BO_3, 20.10 µM of $MnSO_4 \cdot H_2O$, 0.40 µM of $CuSO_4 \cdot 5H_2O$, 0.50 µM of $Na_2Mo_4 \times 2H_2O$, 2.00 µM of $Zn(NO_3)_2 \times 6H_2O$ and 15.00 µM of ferric tartrate. Following the acclimation period, plants were transferred into plastic pots (3.6 L), filled with a mixture containing 8–20 Ø mm expanded clay and sand (50/50 v/v). Plants were subsequently transferred outdoors and irrigated. Over the entire period of treatment, plants grew under an average temperature of 21.3 °C, with an average minimum and maximum temperature of 16.8 °C and 26.2 °C, respectively. The average relative humidity was 76.2% and the average minimum and maximum relative humidity were 54.4% and 93.1% respectively. In addition, there were 7 days of rain for a total of 110 mm (Supplementary Figure S1). Environmental data were collected from the closest weather station located at coordinates 43°42'16''N 10°23'53''E.

Granular fertilizer was applied before the beginning of the experiment. After one month of growth, the average plant height was 34.4 ± 7.7 cm. At the start of the experiment (T_0), each plant was randomly assigned to control (CP) and NaCl treatment group (TP). The experiment was set up according to a completely randomized block design with 5 biological replicates. All plants were watered three times per week with the same volume of water (350 mL). Each TP plant received a cumulative amount of 24 g of NaCl throughout the 27 days of the experiment. At the end of the treatment, the electrical conductivity (EC, dS m^{-1}) of the pot substrate was evaluated following a protocol adapted from FAO (2021). Briefly, substrate samples were dried at 30 °C, grinded and sieved (2 mm porosity). Distilled water was added to each sample in a 1:2 soil: water ratio (w/v) and the samples were mixed for 1.5 h at room temperature (RT) using horizontal shaking. The samples were then left to settle overnight at RT and subsequently the supernatant was filtered. EC was determined using a conductivity meter at 25 °C. The mineral content of the soil solution was also evaluated and expressed as mg kg^{-1} DW. Soil EC was 3–5 times higher in TP pots compared to CP pots (Supplementary Figure S2).

Plant growth measurements

Plant height (cm), stem diameter (mm), and leaves number were measured at the beginning of the experiment and subsequently once a week throughout the entire experimental period. Plant´s apex was initially marked to facilitate the tracking of new leaves formation. Stem height was measured from the apex to the stem collar, while stem diameter was measured 2 cm above the collar with a digital caliper.

Total leaf area was quantified for each plant by measuring a pool of three leaves (one basal, one median and one apical) before the final sampling. The fresh mass (FW) of these leaves was recorded. Leaf area was determined by scanning each leaf and the analysis was performed through ImageJ software (https://imagej.nih.gov/ij/). The total leaf area (A_{total}, cm^2) was estimated according to the formula used in Sharmin et al. (2021):

$$A_{total} = \frac{A_1 + A_2 + A_3}{FW_1 + FW_2 + FW_3} \times FW_{total}$$

where A_{1-3} is the area of each leaf collected for the measure, FW_{1-3} is the corresponding fresh weight and FW_{total} is the total fresh biomass of the entire foliar system.

Plant sampling and relative water content determination

At the end of the experiment, all the plants were harvested. From the start of the experiment the new developed leaves and stem were classified as apical leaves and apical stem, while the previously formed leaves and stem as basal leaves and basal stem. Leaves, stems, woody cutting and roots were collected, and their total fresh and dry weights (FW and DW) were recorded. Five apical and basal leaves were weighed and then dried at 70 °C for mineral content analysis, while two fully expanded leaves randomly selected from both apical and basal portions were used for Relative Water Content (RWC) assessment. For RWC determination, leaf disks with a diameter of 16 mm were collected and weighed to obtain their FW. These disks were then placed in petri dishes filled with MilliQ water and stored at 4 °C in the

dark until there was no further variation in weight (saturated weight – SW). The DW of each sample was determined after drying at 70 °C until a constant weight was achieved. RWC was calculated using the equation:

$$RWC = \frac{(FW - DW)}{(SW - DW)}$$

Apical and basal stem and root samples were also collected and dried for mineral content analysis.

Total chlorophyll content, chlorophyll *a* fluorescence and gas exchanges measurements

Chlorophyll concentration was estimated at the beginning of the experiment and then once a week using a SPAD meter (SPAD502 Plus Chlorophyll Meter, Spectrum, Plainfield, IL, USA). Briefly, five fully expanded leaves were randomly selected from each plant for SPAD measurement. Three readings were taken from each leaf without damaging the plant tissue, and the average of all measurements was calculated. At the end of the experiment, small discs (10 mm) from young and old leaves were collected after taking SPAD meter reading in order to set up a calibration curve. These discs were weighed, and 1 mL of pure methanol (Supelco®, Sigma, Italy) was added for chlorophyll extraction. After incubating in the dark at 4 °C for approximately two days, the samples were analysed using a Tecan Infinite® 200 PRO spectrophotometer. The concentration of chlorophyll a+b was determined by measuring the absorbance at 652 nm (A_{652}) and 665 nm (A_{665}) and calculated according to the equation proposed by Lichtenthaler (1987):

$$Chlorophyll\ a + b\ (\mu\ g/ml) = 1.44 \times\ A_{665.2} + 24.93 \times\ A_{652.4}$$

SPAD values were converted into chlorophyll a+b concentration, expressed as µg g^{-1} FW, using a regression curve ($y = 24.57x - 253.05$; $R^2 = 0.63$).

To evaluate photosystem II efficiency (PSII), chlorophyll *a* fluorescence was measured. At the beginning of the treatment and weekly thereafter, PSII was measured on each plant on a fully expanded median leaf. Before the measurement, leaves were dark-adapted with a leaf-clip for 1 h. A portable pulse-modulated chlorophyll fluorometer (Hansatech FM2, Hansatech Instruments Ltd, Norfolk, UK) was used. The minimum fluorescence yield in the dark-adapted state (F_0) and the maximum fluorescence (F_m) were recorded following a saturating light pulse intensity of 3000 µmol m^{-2} s^{-1} (600 W m^{-2}) emitted by a halogen light source. The maximum quantum efficiency of photosystem II was expressed as Fv/Fm, where Fv represents the difference between F_m and F_0.

Net CO_2 assimilation rate (P_n, µmol CO_2 m^{-2} s^{-1}), leaf stomatal conductance (g_s, mmol m^{-2} s^{-1}), sub-stomatal CO_2 concentration (C_i, ppm) and transpiration (E, mmol H_2O m^{-2} s^{-1}) were measured for each plant on a fully expanded median leaf using a portable photosynthesis system (Ciras-2, PP System International, MA, USA). Readings were collected from 9:00 am to 12:00 am. The instrument was set at 100 mL min^{-1} chamber flow rate, 415 ± 10 ppm CO_2 (ambient concentration), 1000 µmol m^{-2} s^{-1} photosynthetic flux density and ambient temperature and humidity. All the parameters were assessed after exposing the leaves to the new CO_2 concentration for 120–240 s before collecting measurements. Intrinsic Water Use Efficiency (WUE_{int}) was also computed as the ratio between P_n and g_s (WUE_{int}).

Mineral analysis of plant material and substrate samples

Plant samples were dried at 70 °C until a constant weight was achieved. Approximately 0.3 g of plant tissue (leaves, stems, woody stems and roots) was grinded and placed into a 70 mL teflon vessel, followed by digestion in 8 mL of 65% nitric acid using the COOLPEX Smart Microwave Reaction System (Yiyao Instrument Technology Development Co., Ltd., Shanghai, China). The digested samples were allowed to cool, diluted in MilliQ water and then analysed for sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), zinc (Zn), iron (Fe), copper (Cu) and manganese (Mn) concentration using atomic emission spectroscopy (4210 MP-AES, Agilent). Calibration curves were established by measuring a multi-element standard control in a 2% HNO_3 (v/v) solution. *Daucus carota* L. and *Brassica oleracea* L. leaf tissues were used as analytical standard references (WEPAL IPE, Wageningen University, Wageningen, Netherlands). Instrumental parameters (nebulizer flow and viewing position) were optimized for each plant organ and the results were expressed on a dry mass basis (mg kg^{-1} DW).

Evaluation of *aqua1* over-expression

Frozen samples of leaves from WT and over-expressing lines (CP and TP) that were collected at the end of the treatment were grinded in liquid nitrogen using a mortar and pestle. For each sample, an aliquot of around 100 mg was weighed and used for RNA extraction with the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA), following the manufacturer's protocol. RNA quantity and quality were evaluated using Eppendorf BioPhotometer Model 6131 (Eppendorf, Germany) and gel electrophoresis. The Thermo Scientific™ Maxima™ H Minus cDNA Synthesis

Master Mix with dsDNase kit (Thermo Fisher Scientific, USA) was used to convert RNA into cDNA and to remove any residual genomic DNA. RT-qPCR was performed on retro-transcribed samples using the PowerUp™ SYBR™ Green Master Mix kit (Applied Biosystem, USA) and the CFX Connect Real-Time PCR Detection System (Bio-Rad, USA). The expression of *aqua1* gene and two reference genes, *actin 2* (*ACT2*) and *elongation factor 1* (*EF1*), whose expression stability was previously confirmed in poplar (Brunner et al. 2004), were analysed. Three biological replicates from each group were subjected to analysis. The primers used for each gene were: *aqua1 forward* 5'-CCTCCGTGGCATTCTGTATT-3'; *aqua1 reverse* 5'-CAAAGGTCCAGCCCAGTAAA-3'; *ACT2 forward* 5'-CCCATTGAGCACGGTATTGT-3'; *ACT2 reverse* 5'-TACGACCACTGGCATACAGG-3'; *EF1 forward* 5'-AAGCCATGGGATGATGAGAC-3'; *EF1 reverse* 5'-ACTGGAGCCAATTTTGATGC-3'. The *aqua1* primers were designed to evaluate both the expression of the transgene and of the endogenous gene of *Populus alba* L. clone 'Villafranca'. Relative gene expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method and normalized to the WT control group.

Polyphenols analysis

The polyphenols concentrations in roots and leaves of CP and TP of WT, Line 16 and Line 24 were determined according to Vichi et al. (2024). Targeted quantitative analysis of 37 polyphenols was performed. Briefly, 1 g of powdered root tissue and 1 g of powdered leaf tissue were incubated in a 3.35 mL solution of 80% methanol for 1 h at room temperature in the dark. The solution was then centrifuged at 1000 rpm for 30 min in an Allegra 64R-Beckman centrifuge (Beckman, Milan, Italy) and filtered using syringe and cellulose filters (0.2 µm, Millipore, Milan, Italy). Before analysis with HPLC, samples were diluted 1:20 with Milli-Q water (Millipore, Milan, Italy) in 2 mL amber glass screw cap tubes. Polyphenols were quantified using a targeted analytical method with specific standard references to build a standard calibration curve for each analyte. The concentration of each selected metabolite was calculated by interpolation. Standard solutions of the specific polyphenols to be quantified were purchased from Sigma–Aldrich (Milan, Italy) and prepared with serial dilutions from 1 ng mL^{-1} to 512 ng mL^{-1}. Targeted quantitative analyses of selected polyphenols were performed on the extracts by LC-MS/MS using a Sciex 5500 QTrap + mass spectrometer (AB Sciex LLC, Framingham, MA, USA) equipped with a Turbo V ion-spray source and coupled to a mass analysis system. Chromatographic separation was achieved with Phenomenex Kinetex EVO 2 × 100 mm, 5 µm column (Phenomenex, Torrance, CA, USA). The elution was performed using acetonitrile/water 15/85 containing 0.1% formic acid as the mobile phase at a flow rate of 400 µL min^{-1}. Injection volume was of 5 µL and column oven temperature was of 40 °C.

The common source parameters were as follows: nebulization gas (GS1) 50, turbo gas (GS2) 50, curtain gas (CUR) 10, temperature (TEM) 500 °C, ion spray voltage (IS) -4500 V and input potential (EP) 10 V. The compound parameters, including declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP), were adjusted for the selective reaction monitoring (SRM) of each component. The SRM transitions and corresponding compound parameters are listed in Table 1, with multiple reaction monitoring (MRM) used for the highly specific and sensitive acquisition of compounds with complex mixtures.

Statistical analysis

Shapiro-Wilk and Levene's tests were used to check data normality and homogeneity of variances, respectively. Two-way ANOVA was performed for all datasets and means were subjected to Tukey's Multiple Comparison Test ($P < 0.05$). Additionally, Z-scores were calculated for polyphenols data to standardize the values and facilitate comparisons. Heatmaps were generated based on Z-scores to visualize the distribution pattern and determine which polyphenols are better represented in each line. The analysis and visualization were conducted using Microsoft Excel and GraphPad Prism version 8.0.1 software for Windows (GraphPad Software, San Diego, California USA).

Results

The relative over-expression analysis of *aqua1* gene was evaluated on fully expanded basal leaves through RT-qPCR. Line 16 showed a relatively high over-expression (+200-fold-change considering the average of CP and TP) compared to WT plants, as well as Line 24 (+48-fold-change). In addition, Line 16 over-expression was statistically different from WT. No differences were observed in all lines between CP and TP, indicating that the salt treatment had no effect on *aqua1* expression level.

At the beginning of the experiment, all lines were uniform in terms of stem height, diameter and number of leaves (data not shown). After 27 days, transgenic and WT plants were phenotypically indistinguishable (Fig. 1B-D). Measures of FW and DW of all the organs showed a significant growth reduction in TP plants (Supplementary Figure S3). The decrease of stem elongation under salinity followed the order: WT (59%), Line 24 (41%) and Line 16 (26%), and WT was significantly different from Line 16 (Fig. 2A). Leaf area (Fig. 2B) showed a significant reduction under salinity

Table 1 Multiple reaction monitoring (MRM) of selected polyphenols. Compound-dependent parameters for scheduled MRM scan survey, per metabolite: retention time (RT) in minutes (min), precursor mass (Q1) and product mass (Q3), declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) in volts (V)

Polyphenol	RT (min)	Q1	Q3	DP (V)	CE (eV)	CXP (V)
Tyrosol	2.43	137.0	106.0	-78.0	-22.0	-13.0
Cinnamic acid	4.77	147.0	103.0	-65.0	-15.0	-16.0
Hydroxytyrosol	1.92	153.0	123.0	-100.0	-20.0	-17.0
Protocatechuic acid	1.92	153.0	109.0	-86.0	-21.0	-7.0
2-Coumaric acid	3.96	163.0	119.0	-65.0	-18.0	-11.0
3-Coumaric acid	3.63	163.001	119.001	-65.0	-18.0	-11.0
4-Coumaric acid	3.35	163.002	119.002	-65.0	-18.0	-11.0
Vanillic acid	2.86	166.90	108.0	-62.0	-26.0	-13.0
Gallic acid	1.28	168.9	125.0	-75.0	-20.0	-13.0
Caffeic acid	2.80	178.9	135.0	-86.0	-23.0	-11.0
t-Ferulic acid	3.65	193.0	134.0	-62.0	-20.0	-8.0
Hydroxytyrosol α-acetate	3.73	195.1	59.1	-85.0	-24.0	-13.0
Naringenin	5.02	270.9	150.9	-120.0	-25.0	-10.5
Phloretin	5.13	273.0	167.0	-103.0	-38.0	-11.0
Apigenin	5.04	268.9	117.0	-120.0	-49.0	-14.0
Luteolin	4.60	284.9	133.0	-130.0	-44.6	-17.4
Catechin	2.69	289.0	244.9	-108.0	-22.0	-11.0
Epicatechin	2.98	289.001	244.901	-108.0	-22.0	-11.0
Quercetin	4.64	301.0	150.9	-113.0	-38.0	-8.0
Myricetin	2.56	316.9	150.9	-125.0	-33.0	-9.0
Chlorogenic acid	3.97	353.0	151.0	-61.0	-24.0	-9.0
Rosmarinic acid	4.01	359.0	161.0	-70.0	-22.5	-10.0
Piceid	3.69	389.1	227.0	-125.0	-32.0	-11.0
Phloridzin	3.85	435.1	272.9	-135.0	-23.0	-5.0
Kaempferol 7-*O*-glucoside	3.46	447.1	284.9	-158.0	-38.0	-5.0
Kaempferol 3-*O*-glucoside	3.75	447.1	284.1	-202.0	-39.0	-11.0
Quercetin 3-*O*-glucoside	3.16	463.1	300.0	-154.0	-37.0	-5.0
Chicoric acid	3.08	473.1	149.0	-76.0	-30.0	-8.0
Quercetagetin 7-*O*-glucoside	4.42	479.1	316.9	-152.0	-31.0	-14.0
Cynarin	4.09	515.1	191.0	-120.0	-41.0	-7.0
Procyanidin B1	2.80	577.099	288.999	-156.0	-35.0	-11.0
Procyanidin B2	3.52	577.1	289.0	-156.0	-35.0	-11.0
Procyanidin B3	4.55	577.101	289.001	-156.0	-35.0	-11.0
Kaempferol 3-*O*-rutinoside	3.29	593.2	284.9	-138.0	-40.0	-5.0
Tiliroside	3.48	593.201	284.901	-138.0	-40.0	-5.0
Rutin	3.00	609.2	299.9	-154.0	-48.0	-11.0
Quercetin-3,4-*O*-diglucoside	4.77	625.1	270.9	-178.0	-85.0	-12.0

when comparing WT and Line 16 (43% vs. 16.3%) while data of total DW reduction were not significantly different among transgenic lines and WT plants (Fig. 2C).

Total chlorophyll content and chlorophyll *a* fluorescence were monitored during the experiment and the F_v/F_m ratio was calculated as indicator of PSII efficiency (Fig. 3A, C). During the treatment, there was a generalized slight increase of chlorophyll concentration, with no differences between lines and treatments, indicating that salt stress did not induce chlorophyll degradation (Fig. 3A). On the contrary, F_v/F_m ratio showed a clear decrease starting from day 21 in all treated plants (Fig. 3C). In particular, there was a reduction at the end of 17, 27 and 12%, respectively, in WT plants and in over-expressing Line 16 and 24. A loss in net photosynthesis (P_n) and stomatal conductance (g_s) was evident in TP since the first day of treatment, and this gap constantly increased during the whole experimental period (Fig. 3B, D). After 27 days, both g_s and P_n were close to 0 in TP plants. No differences were observed between lines inside each group of treatment. Results for intercellular CO_2 (Ci), transpiration (E) and intrinsic Water Use Efficiency (WUE_{int}) are reported in Supplementary Figure S4. While E followed the same trend observed for P_n and g_s, Ci, after a first decrease in treated plants, showed an increase in the last day of measure, and WUE_{int} was generally higher in salt treated plants during the first weeks. Relative Water Content

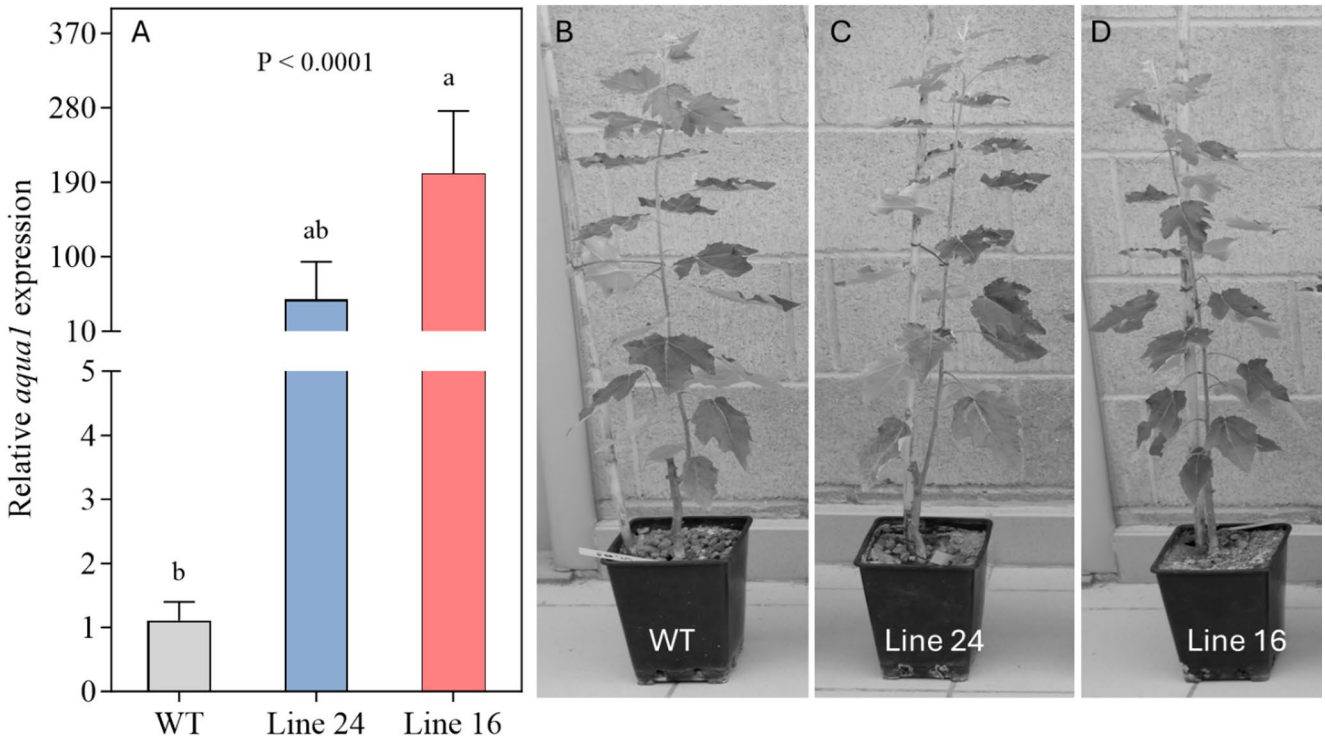

Fig. 1 (**A**) Relative *aqua1* expression. Data are average values of CP and TP leaves of wild-type (WT) and transgenic plants (Line 24 and Line 16). Each bar is the mean + SE of 6 biological replicates (3 CP and 3 TP). Data were analysed with one-way ANOVA followed by Tukey's Multiple Comparison Test ($P < 0.05$). Different letters denote statistically different values. (**B-D**) WT, Line 24 and Line 16 after 27 days of growth under 0 mM NaCl (control conditions)

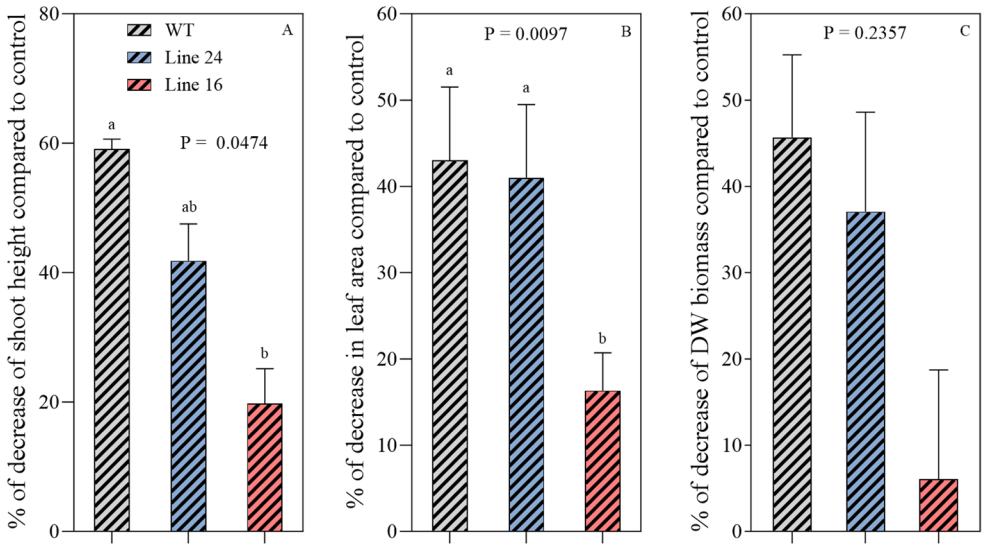

Fig. 2 (**A**) Decrease of shoot height (%), (**B**) leaf area (%), and (**C**) DW biomass compared to respective control plants after 27 days of experiment on WT and over-expressing Line 16 and 24. Each bar is the mean + SE ($n = 5$). Data were arcsin √x transformed, then analysed with one-way ANOVA followed by Tukey's Multiple Comparison Test ($P < 0.05$). ANOVA P-values are reported

(RWC) was higher in Line 16 control plants compared to WT, but not altered in leaves between control and treated groups (Fig. 3E).

Salt treatment determined an increase in Na concentration in TP (Fig. 4). In the roots, transgenic lines showed an accumulation trend that seemed to be negatively correlated to *aqua1* over-expression: the lowest concentration was observed in Line 16, and an intermediate concentration comparable to WT plants was observed in Line 24. Potassium concentration was also affected by the treatment (Fig. 5 and Supplementary Figure S5). We observed a decrease in roots and partially in stems under salinity. On the contrary, values were increased in apical and basal leaves of WT and Line 24 but not in Line 16. Results for the other elements analysed (Ca and Mg for macro- and Cu, Fe, Mn and Zn for

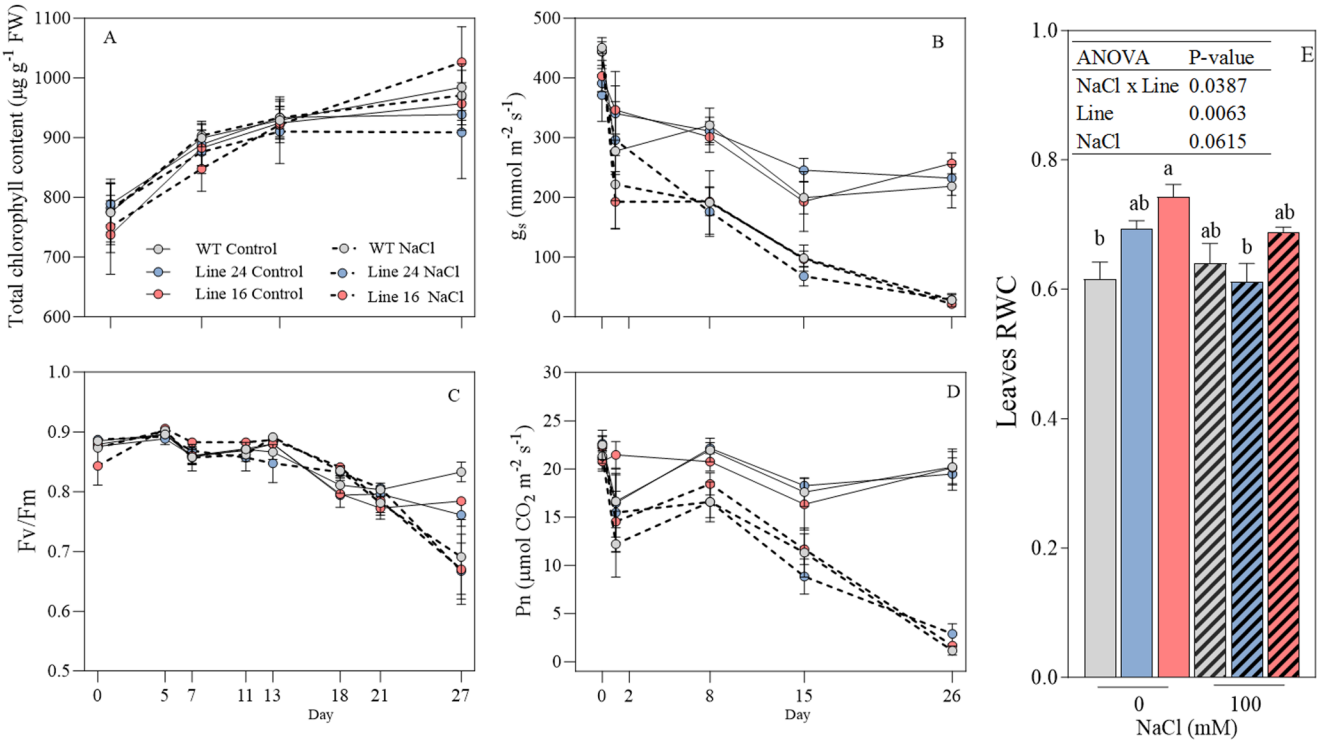

Fig. 3 (**A**) Chlorophyll a + b concentration, (**B**) stomatal conductance, (**C**) PSII efficiency, (**D**) net photosynthesis and (**E**) Relative Water Content (RWC) in leaves of WT and over-expressing Line 16 and 24 plants grown under control (0 mM NaCl) and salinity (100 mM NaCl) conditions. Each value is the mean ± SE ($n = 5$) in panel **A**-**D** and each bar is the mean + SE ($n = 5$) in panel **E**. Data were analysed with 2-way ANOVA followed by Tukey's Multiple Comparison Test ($P < 0.05$). ANOVA P-values are reported in the insert of panel **E** and in Supplementary Table S1 for panels **A**-**D**

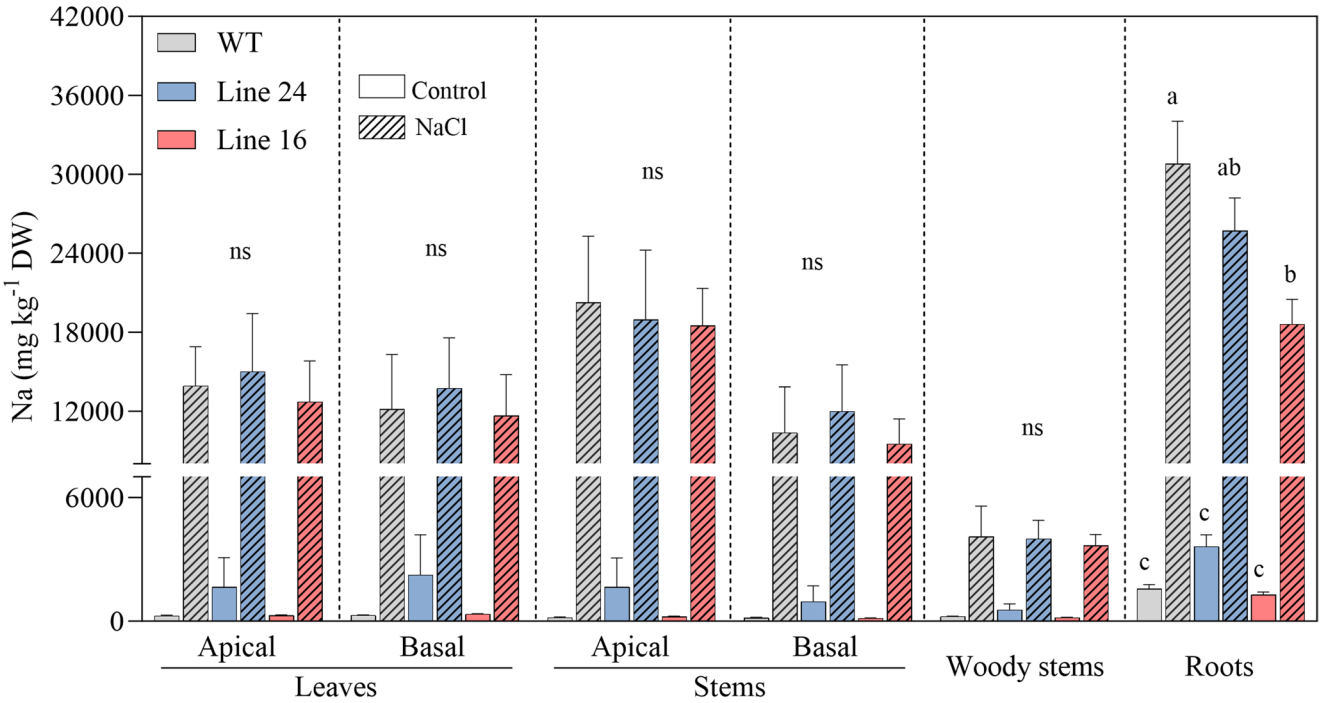

Fig. 4 Sodium concentration (mg kg^{-1} DW) in leaves, stems and roots of WT and over-expressing Line 16 and 24 plants grown under control (0 mM NaCl) and salinity (100 mM NaCl) conditions. Each bar is the mean + SE ($n = 5$). Data were analysed with 2-way ANOVA followed by Tukey's Multiple Comparison Test ($P < 0.05$). ANOVA P-values are reported in Supplementary Table S2. Different letters denote significant different groups; ns = not significant

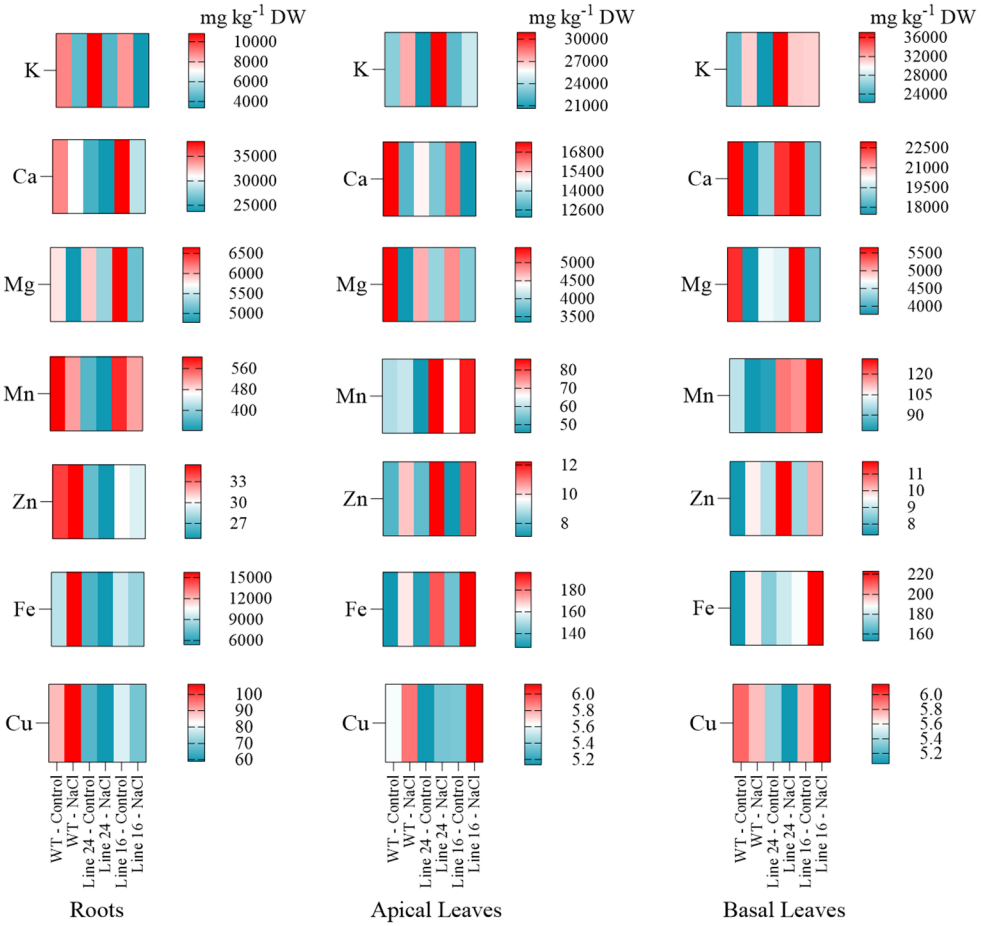

Fig. 5 Heatmap of mineral elements in roots and leaves (apical and basal) of WT and transgenic lines (Line 16 and Line 24) overexpressing *aqua1* under control (0 mM NaCl) and treatment (100 mM NaCl) conditions. Z-scores reflecting the abundance of each mineral element are color-coded with values ranging between relatively low (blue) and high (red) concentrations (mg kg^{-1} DW)

micro-nutrients) are shown in Fig. 5 and in Supplementary Figure S6-11.

There was a decrease in Ca and Mg both in roots and leaves of TP. As far as micro-elements are concerned, we observed a general increase in the root of WT plants in terms of Cu and Fe. On the contrary, both for Line 24 and 16, Fe, Zn, Cu and Mn uptake was increased in the leaves (Fig. 5 and Supplementary Figure S6-S11).

The changes in polyphenols levels in roots under control (0 mM NaCl) and treatment (100 mM NaCl) conditions are illustrated in Fig. 6. Polyphenols of control roots of Line 16 showed an increased concentration compared to the other lines. However, under salinity, the level of polyphenols decreased significantly. Strikingly, Line 16 demonstrated higher levels of four of the measured compounds in mock samples compared to the other compounds, specifically: Piceid acid, Kaempferol 3-*O*-rutinoside, Rosmarinic and Cicoric acid (Fig. 7).

The differential abundance of selected polyphenols in the leaves of WT and the two transgenic lines under control conditions (0 mM NaCl) and salt treatment (100 mM NaCl) is represented in Fig. 8.

According to the heatmap, 7 out of 13 recorded polyphenols (Kaempferol 3-*O*-rutinoside, Hydroxytyrosol, Chlorogenic acid, Procyanidin B1, Procyanidin B3, Catechin and Epicatechin) were present at high concentration under control conditions in Line 16, while their level was clearly lower under stress. The remaining 6 polyphenols maintained a stable profile, showing little to no variation between control and treatment conditions. In Line 24, 9 compounds showed a relatively high concentration under control conditions (Chlorogenic acid, Procyanidin B1, Procyanidin B3, Catechin, Epicatechin, Vanillic acid, 2-Coumaric acid, t-Ferulic acid and Protocatechuic acid) and of them, 7 compounds (Procyanidin B1, Procyanidin B3, Catechin, Epicatechin, Vanillic acid, 2-Coumaric acid and Protocatechuic acid) showed a reduction under stress, whereas the 3 other compounds (Kaempferol 3-*O*-rutinoside, Chlorogenic acid and t-Ferulic acid) remained high under stress, showing little change. Additionally, an increase was observed in the case of other 3 compounds (Kaempferol 3-*O*-rutinoside, Kaempferol 3-*O*-glucoside and Kaempferol 7-*O*-glucoside) that were lower under control conditions. In contrast, WT plants showed a more stable profile compared to the two transgenic lines, with few changes between control and salt

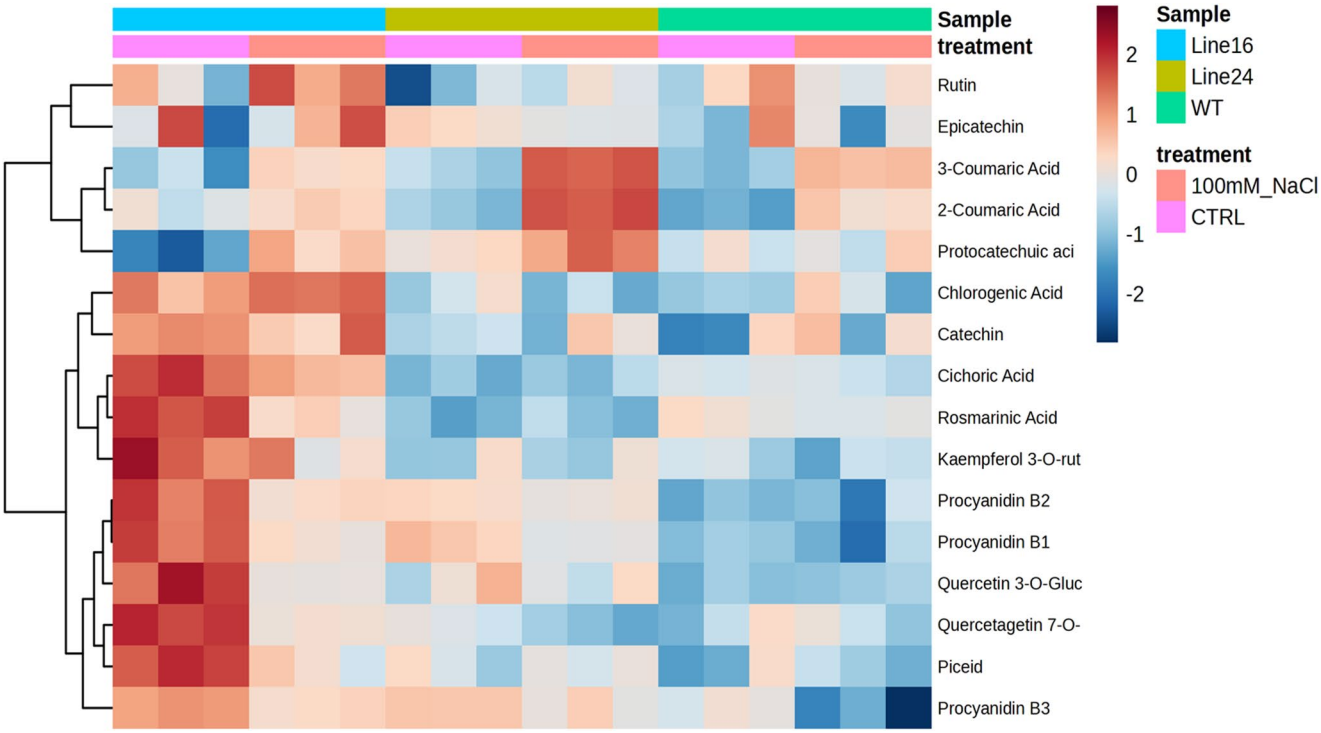

Fig. 6 Heatmap of detected polyphenols in roots ($n=3$) of WT and transgenic lines. Lines over-expressing *aqua1* (Line 16 and Line 24) and WT leaves under control (0 mM NaCl) and treated (100 mM NaCl) conditions. Z-scores reflecting the abundance of each compound are color-coded with values ranging between −2 and 2; negative values are in blue scale and positive values are in red scale

stress conditions. This indicates that WT plants exhibited a more consistent response with minor shifts in the levels of several polyphenols under salt stress.

Discussion

Salinity has detrimental effects on plant growth and survival. The increasing accumulation of salt in soil causes an increase in salt uptake by plants. Excessive salt is toxic for cell metabolism, and it is the cause of nutrient imbalances, metabolic disorders and oxidative stress. In salt-sensitive species, main consequences are related to a reduction in photosynthetic performances, leaf necrosis and abscission and a general reduction of plant growth or increase of mortality (Kibria and Hoque 2019).

Aquaporins are membrane-integral water channel proteins that allow water, metalloids, gases and other small neutral solutes to pass across cellular membranes (Gomes et al. 2009). It has been observed that salinity can induce the up-regulation of aquaporin genes, resulting in increased salt stress tolerance by the accumulation of K^+, Ca^{2+} and proline in *Arabidopsis* (Sade et al. 2010; Gao et al. 2010). Moreover, other studies have also shown that the over-expression of specific aquaporins by transgenesis has improved salinity tolerance, as in the case of the aquaporin SlTIP2;2 from *Solanum lycopersicum* L. in *Arabidopsis* (Xin et al. 2014) and the TaAQP8 from wheat in tobacco (Hu et al. 2012).

This study investigated the role under salinity of *aqua1*, a TIP aquaporin from *Populus* × *canadensis* Moench clone 'I-214', in over-expressing plants of *Populus alba* L. clone 'Villafranca'. The lines tested were previously transformed by Ariani et al. (2016), where the cDNA of the gene (771 nucleotides), codifying for a 257 amino acids protein, was introduced inside plant genome under the action of a strong promoter. In Ariani et al. (2016, 2019), it is explained that *aqua1* genomic sequence contains two exons and one intron and the protein is mainly localized in the tonoplast. Previously, the over-expression of *aqua1* in *Populus alba* L. lines induced an increase in growth rate and water use efficiency under high Zn conditions (Ariani et al. 2016). Throughout our experiment, these lines were assessed for their physiological and biochemical responses to stress. At the end of the experiment, substrate electrical conductivity was measured for quantifying the level of the stress applied (Supplementary Figure S2). The EC values of 3.5–4.9 dS m^{-1} in treated plants were in line with the classification of soil considered saline ($EC_e > 2$ dS m^{-1}) (Daliakopoulos et al. 2016).

Salt stress had an impact on all morphological parameters measured (shoot height, leaf area and dry biomass) as well as leaves RWC in WT treated plants (Figs. 2 and 3E). Although these plants showed a reduction in growth,

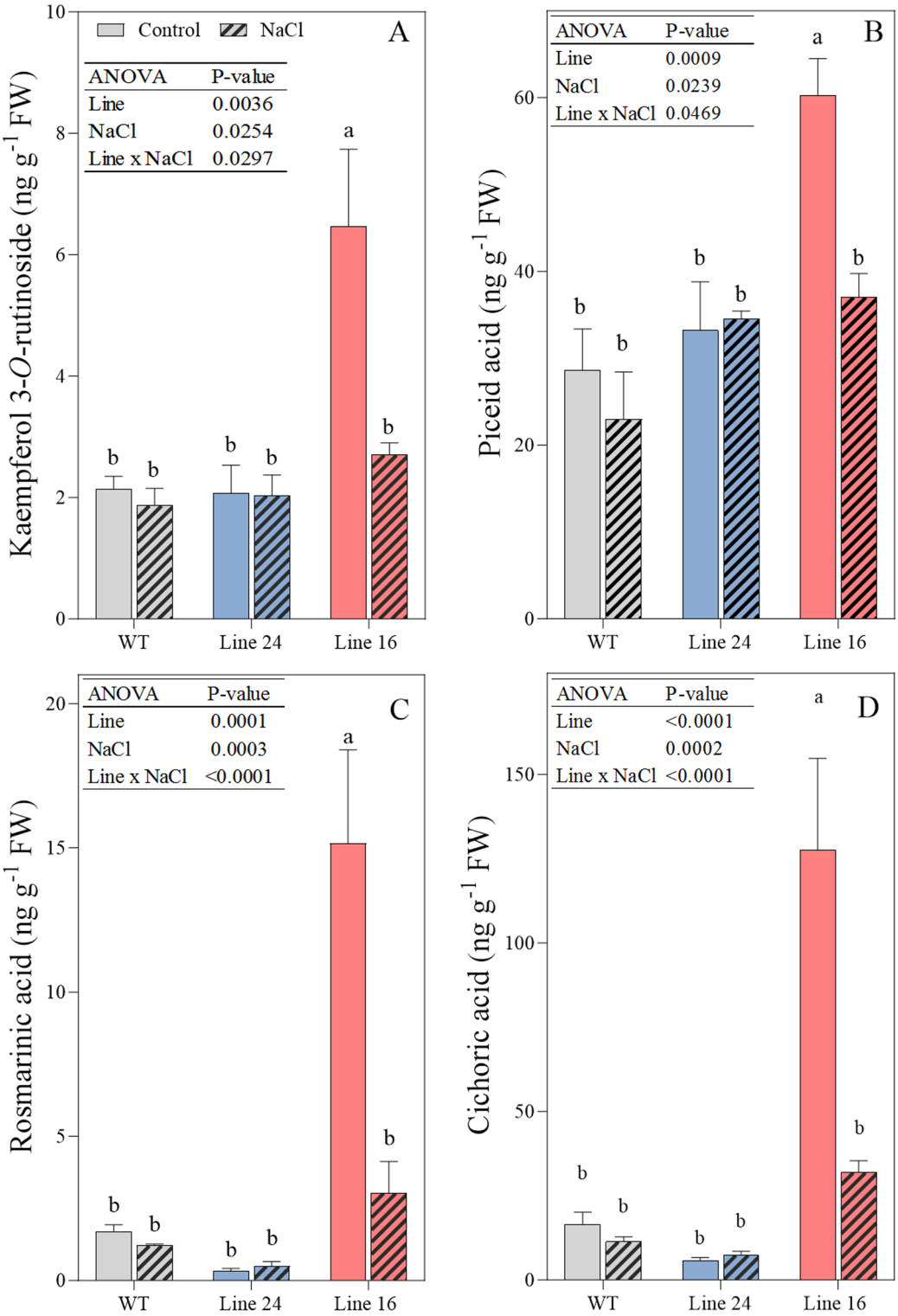

Fig. 7 Concentrations (ng g^{-1} FW) of (**A**) Kaempferol 3-O-rutinoside, (**B**) Piceid acid, (**C**) Rosmarinic acid and (**D**) Cichoric acid in roots of WT and over-expressing Line 16 and 24 plants grown under control (0 mM NaCl) and salinity (100 mM NaCl) conditions. Each bar is the mean + SE ($n = 3$). Data were analysed with 2-way ANOVA followed by Tukey's Multiple Comparison Test ($P < 0.05$). ANOVA P-values are reported in the inserts and different letters denote significant different groups

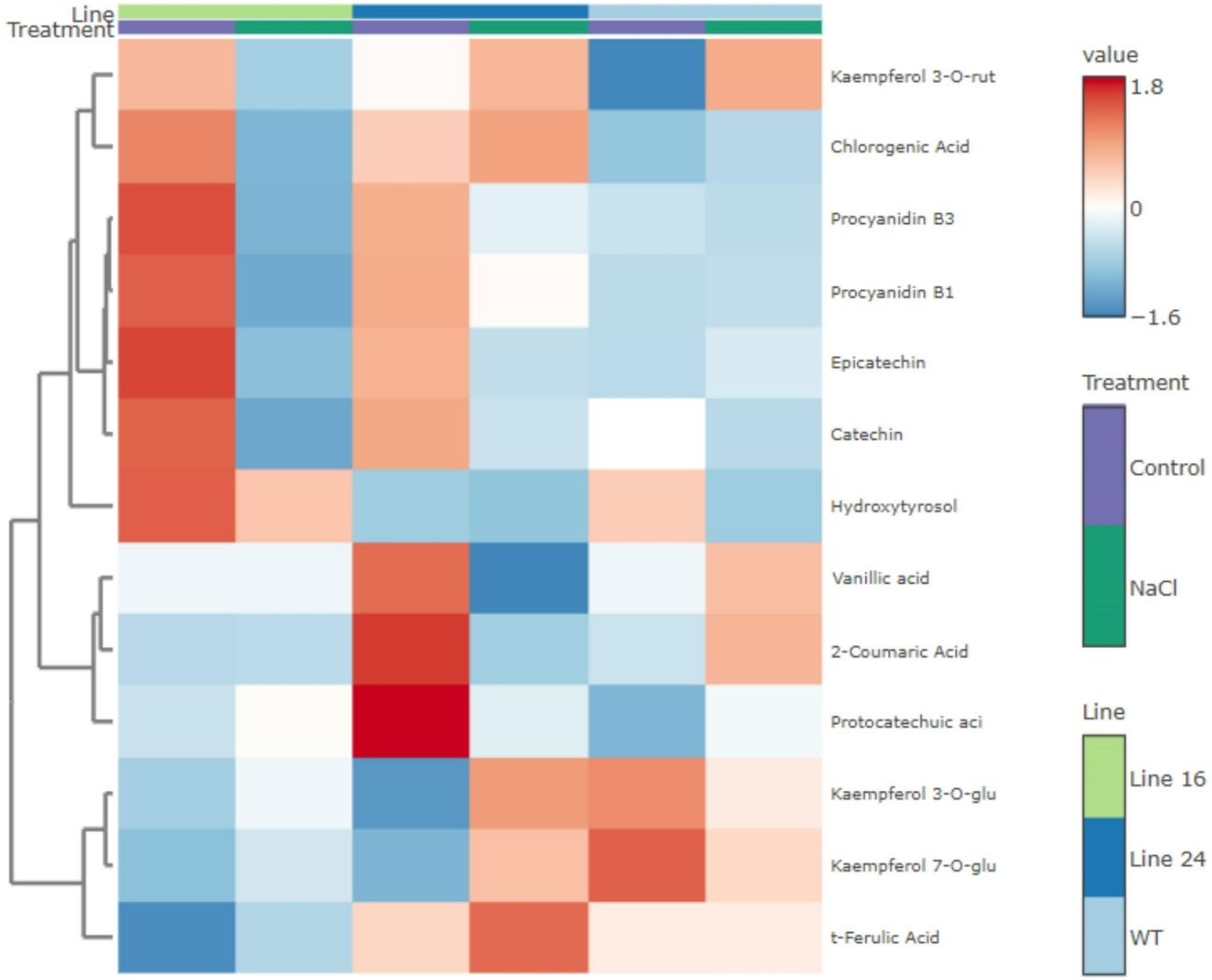

Fig. 8 Heatmap of detected polyphenols in leaves of WT and transgenic lines (Line 16 and Line 24) under control (0 mM NaCl) and treated (100 mM NaCl) conditions. Z-scores reflecting the abundance of each compound are color-coded with values ranging between −1.6 and 1.8; negative values are in blue scale and positive values are in red scale

their ability to survive under a prolongued exposure to 100 mM NaCl indicated a certain level of salt-tolerance of clone 'Villafranca', confirming the adaptive variability observed in *Populus alba* L. in response to salinity and for which this group of poplar plants is of interest in salt stress studies (Sixto et al. 2005; Chen and Polle 2010; Della Maggiora et al. 2023).

The over-expressing *aqua1* lines showed a significantly different response under salinity compared to WT plants. At morphological level, the relative reduction in shoot elongation and total leaf area was lower in Line 16, the one with the highest over-expression, if compared to the reduction observed in WT plants (Fig. 2A-B). At physiological level, salinity did not significantly alter total chlorophyll content in all lines and conditions (Fig. 3A) but a significant decrease in terms of F_v/F_m ratio was observed at the end of the experiment (Fig. 3C). Gas exchanges parameters (P_n, g_s and E) started to decrease in treated plants from the first day of treatment and they were all close to 0 at the end (Fig. 3B, D and Supplementary Figure S4B). The photosynthetic decline was consistent with the Na accumulation observed in treated plants (Fig. 4), as also observed in Della Maggiora et al. (2023) in poplar 'Marte' clone exposed to NaCl treatment.

Interestingly, we observed a differential Na concentration in the roots of treated plants. Na concentration seemed to be negatively correlated with *aqua1* over-expression in comparison to WT plants, with Line 16 showing the lowest concentration. As reported by Ruiz-Lozano et al. (2012), aquaporins confer salt stress tolerance primarily by regulating water uptake and its distribution across different plant organs. Additionally, Na^+ and K^+ balance is crucial

for the plant health and Munns et al. (2006) reported that enhanced K^+ uptake and Na^+ exclusion are key mechanisms to enhance the salinity tolerance. This mechanism is well discussed in *Arabidopsis* plants, where over-expression of specific aquaporin lead to higher accumulation of K^+ and Ca^+ and reduced accumulation of Na^+ in roots under salinity (Gao et al. 2010). Under high salinity stress, *P. euphratica* maintained an optimal Na^+/K^+ ratio by restricting the net Na^+ uptake and transport from roots to shoots and by maintaining higher K^+ uptake and transport (Chen et al. 2003; Sun et al. 2009).

The over-expression in *aqua1* Line 16 demonstrated a decreased Na^+ accumulation in roots, suggesting that *aqua1* may influence sodium movement through improved water flux, vacuolar compartmentalization and by maintaining ionic gradient. This results in the dilution of Na^+ and its restricted apoplastic flow, ultimately contributing to mineral element homeostasis under stress.

The main role of aquaporins is to regulate the internal redistribution of mineral nutrients like K^+ by transporting them from the endoplasmic reticulum to the plasma membrane via the Golgi apparatus before reaching the vacuole (Maurel et al. 2008). As reported by Hachez et al. (2014), the link between K^+ and aquaporins is a key mechanism for controlling water movement, osmotic potentials, and mineral element intercellular transport in plants. Our transgenic lines showed a decreased K^+ in roots, but K^+ accumulation significantly increased in apical and basal leaves, compared to WT under salinity, confirming the role of *aqua1* to promote translocation of K^+ in the apical part through the water flux.

In addition, there was a decrease in Ca and Mg both in roots and leaves of treated plants (Fig. 5 and Supplementary Figure S6-7), probably linked to the reduction in stomatal conductance and transpiration (Sharmin et al. 2021). We observed a general increase in the levels of Zn, Cu and Fe in the soil (data not shown) that could be attributed to the reduced mobility of these elements induced by salinity (Evelin et al. 2019). However, these differences were not observed at root level between control and treated plants (Fig. 5 and Supplementary Figure S8-11). Moreover, even if Zn, Fe and Mn uptake seemed to be unaltered, there was an increase for all these elements in leaves, that could be explained by their role, mainly at enzymatic level, in mitigating salt stress effects (Rahman et al. 2016; Hussain et al. 2018).

Among the defense mechanisms against salinity, secondary metabolites, particularly polyphenols or phenolic compounds (PCs), plays a crucial role in enhancing plant resistance to stresses (Šamec et al. 2021; Elshafie et al. 2023). Secondary metabolites are involved in the complex interactions between plants and their environment (Erb and Kliebenstein 2020; Elshafie et al. 2023), affecting growth and development (Sharma et al. 2019; Landi et al. 2020; Hasanuzzaman et al. 2020). Qaderi et al. (2023) discussed the importance of polyphenols in the light of climate change describing their ability to enhance plant tolerance and adaptation to drought, high temperatures and excessive exposure to UV-light. Considering that salinity stress was not addressed, this study sheds light on the variations of secondary metabolites under salt stress conditions, more specifically polyphenols in roots and leaves. Overall, the level of polyphenols in roots remained stable across both control and treatment conditions. However, four compounds (Piceid acid, Kaempferol 3-*O*-rutinoside, Rosmarinic acid and Cichoric acid) demonstrated significantly higher levels in Line 16 under control conditions compared to other compounds and lines under study. The observed increase of these specific polyphenols in transgenic Line 16 compared to WT under control conditions could suggest that *aqua1* over-expression may enhance the plant's ability to produce important secondary metabolites and the decline of their concentration under stress suggests that these transgenic lines may have redirect the resources from production of secondary metabolites towards the important metabolic functions required for stress tolerance. This pattern is in line with the known response of plants to abiotic stress, where the expression of certain stress related genes often leads to an altered profile of metabolites (Noman and Azhar 2023). In contrast to the relatively stable profile of polyphenols observed in roots, leaves displayed a more dynamic response to salinity stress. Line 16, showing the highest over-expression of *aqua1*, presented a distinct shift in its polyphenolic content between control and treatment conditions. Several polyphenols were abundant under control condition and markedly reduced under stress, indicating a redistribution of metabolic resources in response to salinity. This is in line with previous studies, such as in Kravchik and Bernstein (2013), which stated that plant responses to salt stress are highly complex, particularly at organ and cell level, and different ROS scavenging enzymes respond differently across different plant organs, developmental stages and cells age. In our study, *aqua1* over-expression regulates polyphenols concentration by increasing their amount in leaves and in roots of control Line 16. Alavilli et al. (2016) demonstrated that aquaporin over-expression increases stress tolerance to high salt and high osmotic stresses. This mechanism is determined through higher activities and/or expression of ROS scavenging enzymes and osmoprotectant biosynthetic genes. Similarly, Hu et al. (2012) and Guo et al. (2022) showed a reduction of H_2O_2 accumulation when aquaporin genes *TaAQP8* and *GhTIP2* were over-expressed in wheat and cotton, respectively. In our study it seems that polyphenols play a key role in polar response to salinity. In fact, in

Line 16, the polyphenols reduction observed under salinity at root and shoot level is also correlated to the plant health status by promoting overall growth.

In conclusion, our results suggest that *aqua1* was not directly involved in primary mechanism of salt stress responses, but played a role in promoting general plant growth, which contribute to overall stress resistance. Plants possess the highest number of aquaporins among all living organisms. These aquaporins are divided into several subfamilies and distributed across all sub-cellular compartments. In poplar, 55 aquaporins coding genes have been identified (Gupta and Sankararamakrishnan 2009). Aquaporins are involved in many important essential functions in plants, with some playing an important role in plant tolerance to salt stress, making them potential targets for genetic modification. However, the presence of many genes encoding similar subgroups of isoforms in the poplar genome increases the complexity of the analysis focused on specific genes.

To our knowledge this is the first work where the role of a specific poplar TIP aquaporin is characterized in plants under salinity. Our study provides comprehensive information about the role of *aqua1* in Na homeostasis and gives information about the metabolic profile of poplar under salt stress. In future, it would be interesting to investigate how *aqua1* over-expression could activate compensatory mechanisms, both in terms of expression of other aquaporins and at post-translational and cellular localization level. Additionally, research should focus on tissue specific metabolic adjustments to enhance stress tolerance in plants.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10725-025-01353-z.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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