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Differences in mineral and osmotic balances enhance zinc translocation in an aquaporin overexpressing poplar

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ABSTRACT

Zinc (Zn) is an essential micronutrient for plants, but it is toxic beyond a certain threshold. *Populus alba* (L.) 'Villafranca' clone is known for its good tolerance to high Zn concentration compared to other poplar species. A line of this species overexpressing the tonoplast intrinsic aquaporin *AQUA1* gene has showed an improved tolerance to Zn excess in comparison to the wild-type (wt) line. The aims of this work were to: 1) verify if *AQUA1* plants can uptake Zn more efficiently after a longer period of exposure; 2) evaluate if a higher Zn uptake in transgenic lines can have negative effects; 3) assess Zn competing elements (iron and manganese), soluble sugars, osmolytes, and potassium to investigate differences in water and osmotic homeostasis between lines. Under Zn excess, *AQUA1* plants showed a twofold Zn translocation factor and a higher xylem sap Zn concentration than the wt plants. Transgenic plants preferentially allocated Zn in aerial biomass and this different behaviour matched with modified manganese and iron balances suggesting that the increased Zn uptake might be related to a decrease in iron transport in the transgenic line. Moreover, a higher instantaneous water use efficiency in control conditions and an increase in bark soluble sugars under Zn excess could allow a higher resistance of *AQUA1* plants to the water and osmotic perturbations caused by Zn. Indeed, the Zn excess increased the xylem osmolyte content only in wt plants. Further investigations are required to understand the role of *AQUA1* in osmotic regulation.

1. Introduction

Zinc (Zn) is an essential element for plant nutrition; it activates enzymes involved in carbohydrate metabolism, maintenance of cell membrane integrity, protein synthesis, auxin regulation, and pollen formation (Broadley et al., 2007; Marschner, 2012). Zn uptake in plants is influenced by several factors such as the type of cultivation media, plant species and variety, presence of arbuscular mycorrhiza (Garg and Kaur, 2012; Kaur and Garg, 2021), as well as by the irrigation level (Disante et al., 2014). Zn deficiency causes several issues such as stunted growth, development of small leaves, shoot desiccation, and interveinal chlorosis (Barker and Pilbeam, 2015). Zn is toxic beyond a certain level, but a toxicity threshold is difficult to set since this value varies among plant species. Generally, 15–20 mg Zn kg^{-1} of leaf dry matter is considered the minimum concentration for crop growth while the Zn toxicity threshold that might induce visible leaf symptoms is set at 300 mg kg^{-1} (Marschner, 2012).

Zn is adsorbed as a divalent cation and is chelated in roots by organic ligands before the translocation via xylem toward the shoots (Broadley et al., 2007). Root uptake and xylem transport are particularly efficient in plants able to hyperaccumulate Zn, as well as the metal sequestration ability to withstand the toxicity symptoms (Gupta et al., 2016). The heavy metals can also cause perturbations in plant water and osmotic status (Rucińska-Sobkowiak, 2016). Among other, Kasim (2007) and Ghnaya et al. (2010) found in *Phaseolus vulgaris* and *Brassica napus*, respectively, that Zn affected the plant water homeostasis inducing a lower water content in all plant organs. Thus, a parameter to consider in selecting hyperaccumulating plants might be also their capacity of dealing with water and osmotic perturbations caused by the heavy metal accumulation in plant tissues.

Transmembrane water flow in cells is facilitated by aquaporins (AQPs), membrane channels which also allow the passage of gases,

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H_2O_2, and metalloids (Bienert and Chaumont, 2014; Kaldenhoff, 2012; Maurel et al., 2015). Transgenic plants overexpressing the AQP genes has been shown to be more tolerant to suboptimal environmental conditions (Banerjee and Roychoudhury, 2020). Indeed, AQPs affect both water uptake in roots and the regulation of stomatal conductance, considerably determining the plant water balance (Maurel et al., 2016). Heavy metals decrease the water uptake by roots and the short distance transport of water across plant tissues mostly by acting on AQP expression at different levels, from the gene transcription to the protein localization and activity, usually decreasing AQP activity (review by Vats et al., 2021). However, a clear mechanistic role of AQPs under heavy metal excess is yet to be understood (Vats et al., 2021).

Zn effect has been studied in *Populus* spp. by several authors (Chen et al., 2014; Di Baccio et al., 2003, 2005; Stolárik ová-Vaculíková et al., 2015; Todeschni et al., 2011). Di Baccio et al. (2011) highlighted a strong downregulation in both roots and leaves of a TIP aquaporin (*AQUA1* gene) under 1 mM Zn concentration in *P.* × *euramericana* 'I-214' clone. Romeo et al. (2014) investigated the effect of 1 mM of Zn (65 mg kg^{-1}) on four poplar clones, reporting *P. alba* 'Villafranca' clone as the most tolerant among the tested species. This clone showed the highest Zn content within plant organs without visible toxic effects. Thus, on the same clone, Ariani et al. (2016) studied the effect of *AQUA1* overexpression reporting an improvement of intrinsic water use efficiency under Zn excess compared to the wt plants, suggesting the involvement of this AQP in the water homeostasis under heavy metal uptake. Ariani et al. (2019) reported that Zn treatment modified the poplar AQUA1 localization and its post-translational regulation in Arabidopsis protoplasts, probably leading to a reduction in water flow and allowing osmotic adjustments. Indeed, the connection between Zn and osmotic adjustments has been already highlighted under drought in sunflower (Jan et al., 2022) as well as the relation between osmotic adjustments and AQPs through a meta-analysis study (Ren et al., 2021). Thus, the improved performances in water use efficiency of *AQUA1* overexpressing poplars under Zn excess found by Ariani et al. (2016) deserve to be deepened evaluating the possible effect of this AQP in water flow and osmotic adjustments.

The osmotic adjustment consists in a decrease of the osmotic potential arising from the net accumulation of osmotically active solutes; this mechanism allows the cell turgor maintenance under drought stress (Blum, 2017). The compounds mainly involved in the osmotic adjustment are soluble sugars, inorganic cations such as potassium (K) and calcium (Ca), proline, and glycine-betaine (Turner, 2018). Heavy metal stress has been shown to trigger osmotic adjustments also related to the soluble sugar accumulation (Rucińska-Sobkowiak, 2016) and modifications of other mineral nutrients such as manganese (Mn) and iron (Fe) (Socha and Guerinot, 2014). Moreover, Traversari et al. (2020) highlighted in *P. alba* 'Villafranca' clone an important role of soluble sugars in the osmotic potential maintenance under drought, suggesting the involvement of carbohydrate metabolism in stress response at plant tissue level.

Considering all these aspects, the aims of this work were to: 1) verify if the transgenic lines overexpressing *AQUA1* are able to uptake Zn more efficiently under a longer period of Zn excess; 2) evaluate if a higher Zn uptake in transgenic lines can have negative effects; 3) measure the soluble sugars, osmolytes, K, Fe, and Mn changes in wt and *AQUA1* tissues to investigate possible differences in mineral and osmotic relations and thus the involvement of *AQUA1* gene in water and osmotic balances under Zn excess.

2. Materials and methods

2.1. Plant growth and metal treatment

Two lines of *Populus alba* (L.) 'Villafranca' clone were used in this experiment: wild-type (wt) and line 16, described by Ariani et al. (2016). This modified line overexpressed a tonoplast intrinsic aquaporin (TIP) gene (GenBank: GQ918138, homologue of *AtTIP1;1*). The plants derived from *in vitro* culture were acclimatized and maintained as described by Ariani et al. (2016). Briefly, after 3 weeks of acclimatation to the *in vivo* conditions in perlite, plants were transferred in expanded clay in hydroponics. After an acclimation period in hydroponics (average plant height was 82.6 ± 6.81 cm in wt line, 82.9 ± 6.21 cm in *AQUA1* line), control plants (CPs) were supplemented with full strength Hoagland's solution at pH 6.2 (Arnon and Hoagland, 1940) containing 1 μM Zn, while treated plants (TPs) were supplemented with Hoagland's solution at pH 6.2 containing 1 mM Zn. Zn was supplied as Zn nitrate hexahydrate ($Zn(NO_3)_2{\cdot}6H_2O$, Merck KGaA, Darmstadt, Germany), and Fe as Fe-tartrate instead of Fe-EDTA to avoid metal chelation. The Hoagland's solution was renewed every 6 days. From the start of the experiment the new developed leaves were classified as new leaves (NL) while the previous formed leaves as old leaves (OL) to verify a possible different accumulation of Zn in the newly produced biomass.

2.2. Photosystem efficiency, gas exchanges, and plant water relations

Photosystem II efficiency was determined on dark-adapted (15 min) fully expanded NL (leaf plastochrone index between 6 and 7, following the procedure reported by Erickson and Michelini, 1957), using a portable pulse modulated chlorophyll fluorometer FMS2 (Hansatech Instruments Ltd, Norfolk, UK). The measures were taken on the leaf surface exposed to an excitation light intensity of 3000 μmol m^{-2} s^{-1} (600 W m^{-2}) emitted by a halogen light source. Leaf stomatal conductance (g_s, mmol m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), and net CO_2 assimilation rate (A, μmol CO_2 m^{-2} s^{-1}) were weekly measured on fully expanded NL (leaf plastochrone index between 7 and 8) between 2.00 and 3.00 p.m. with a portable photosynthesis system (Ciras-2, PP Systems, Amesbury, MA, USA) operating at 100 mL min^{-1} flow rate, 400 ± 1 Pa ambient CO_2, and a photosynthetic flux density of 1000 μmol m^{-2} s^{-1}. Instantaneous water-use efficiency (WUE*i*, mmol CO_2 mol H_2O^{-1}) was determined as instantaneous leaf transpiration efficiency, A/E. Midday leaf and stem water potentials (leaf Ψ_w and stem Ψ_w, MPa) were measured on two fully expanded NL for each plant (leaf plastochrone index between 5 and 7) with a Scholander pressure bomb. For stem Ψ_w determination, the leaves were covered with aluminium foils at least 30 min before the measurements to allow the equilibration between leaf and stem Ψ_w.

2.3. Samples collection and preparation

After 73 d of treatment, all plants (n = 16) were randomly sampled. The plant height, leaf number, stem diameter, and leaf area (cm^2) were measured. Leaf relative water content (RWC) was determined on two fully expanded NL (leaf plastochrone index between 10 and 11) for each plant. The leaves were cut, weighed for fresh weight (FW) determination, then they were rehydrated placing the petioles in distilled water overnight at 4 °C in the dark in plastic bags to determine the turgid weight (TW). Then, leaves were finally dried 48 h at 105 °C for the dry weight (DW) measure.

The leaf RWC was calculated as follows:

$$RWC = 100 \times (FW - DW)/(TW - DW).$$

The NL that remained after leaf RWC and water potential measurements were divided from the OL and frozen in liquid nitrogen and stored at − 80 °C for the biochemical analyses. A portion of NL and the OL were also directly oven dried at 60 °C.

Xylem sap was collected from a portion of stem (≈1 m) under the apex. The bark was removed and immediately frozen and stored at − 80 °C or directly oven dried. Then, xylem sap (about 2 mL) was extracted applying negative pressure by the vacuum method (Alexou and Peuke, 2013) and immediately frozen. After sap extraction, wood samples were frozen and stored or oven dried.

A stem portions (1 cm) was excised at 25 cm from the collar to determine the FW. It was immerged in water overnight under vacuum, weighted to determine the TW, and dried 48 h at 105 °C to determine the DW.

Then, stem RWC was calculated as reported for leaves.

Finally, the roots were washed in 10 mM $CaCl_2$ and in deionized water to remove the adsorbed Zn and oven dried.

NL, bark, and xylem samples stored in − 80 °C were freeze-dried at − 80 °C under vacuum and reduced to a fine powder with an Ultra Centrifugal mill ZM 200 (Retsch, Haan, Germany). The powder was used for the quantification of osmotically active molecules and soluble sugars. NL, OL, bark, xylem, and root oven dried samples were used for mineral element analysis.

2.4. Mineral element analyses

Zn, Mn, K, and Fe concentrations in OL, NL, bark, xylem, and roots were quantified by atomic absorption spectrometer (AAnalyst 200, PerkinElmer, Waltham, MA, USA). The sample powder (200 mg) was digested with 5 mL of HNO_3 followed by 1 mL $HClO_4$. The resulting solution was filtered and diluted with Milli-Q H_2O and then analysed. Moreover, Zn, Mn, K, Fe, and Ca were directly analysed in the xylem sap using the atomic absorption spectrometer described above and results were expressed in mg L^{-1}.

The Zn translocation factor was calculated using the Zn content, obtained from the Zn concentration and the plant biomass, as follows:

$$\text{Translocation factor} = \frac{[\text{Zn}]\text{shoot}}{[\text{Zn}]\text{root}}$$

where [Zn]shoot was the Zn content obtained from the Zn content within the leaves plus the Zn content within the stem.

The Zn distribution in root and shoot as a percentage of total Zn in plant biomass was also calculated.

2.5. Determination of osmotically active molecules and soluble sugars

Soluble carbohydrate analysis was performed on freeze-dried powder following the procedure reported by Traversari et al. (2020). Briefly, the sugar content was determined by high performance liquid chromatography equipped with a SHODEX SUGAR Series SC 1011, 8 × 300 mm column (Showa Denko K.K., Tokyo, Japan), which was preceded by a pre-column Guard Pak Insert Sugar Pak II (Waters, Antwerp, Belgium). Milli-Q water was used as the mobile phase with a flow rate of 0.5 mL min^{-1}. Soluble sugars were identified with a refractive-index detector (LC-30 RI, PerkinElmer, Waltham, MA, USA). Carbohydrate standards were used for the corroboration of identified sugars while sorbitol was used for normalizing sugar concentrations (Merck KGaA, Darmstadt, Germany).

Osmotically active solutes were determined following the procedure proposed by Arend and Fromm (2007). Freeze-dried powder (4 mg) was suspended in 250 μL of Milli-Q H_2O, vortexed, and sonicated for 10 min. Samples were centrifuged at 10,000 *g* for 5 min and the supernatant was analysed by a freezing point osmometer (Osmomat 030, Gonotec, Germany). Results were expressed in mOsm g^{-1} DW. Moreover, osmotically active solutes were determined directly in the xylem sap and results were converted in osmotic potential (MPa) by Van't Hoff equation.

The sums of contributions to the osmotic potential of sucrose, glucose, and fructose ($\Psi_{\pi(SS)}$, MPa) were determined as reported by Traversari et al. (2020):

$$\Psi_\pi = RT \times RDW \times C$$

where RT is $-0.002479\ m^3\ MPa\ mol^{-1}$ at 25 °C, RDW is the relative dry weight at saturation (kg m^{-3}), calculated considering the TW of leaves, bark, and xylem; C is the molar concentration of solutes (mol kg^{-1}). The $\Psi_{\pi(SS)}$ and the contribution of K and Ca to the Ψ_π ($\Psi_{\pi(ME)}$) in xylem sap (kPa) were directly calculated using the Van't Hoff equation at 25 °C.

2.6. Statistical analysis

The experiment was set up in a completely randomized design. Data were tested for normal distribution using D'Agostino-Pearson K^2 test and eventually transformed before the analysis of variance (ANOVA). The effects of line and Zn treatment were evaluated with a two-way ANOVA. *Post hoc* analysis was conducted using a Fisher's LSD test ($P < 0.05$). All the statistical analyses and graphs were done with Prism 10 software (GraphPad, La Jolla, CA, USA). Correlation matrixes were realized using R studio "corrplot" package.

3. Results

3.1. Plant physiological status

After 73 d of Zn exposure, plants did not show any symptom of chlorosis or stunted growth. Zn excess did not affect the leaf area, stem diameter nor plant height of both wt and *AQUA1* lines (Online Resource Table S1). In control conditions, F_v/F_m parameter was higher in *AQUA1* line than in wt plants, but it became equal under Zn excess (Table 1). The other photosystem II efficiency parameters (NPQ, ETR) were not affected by the genetic modification or the Zn treatment. Despite A was equal between lines, both g_s and E resulted lower in *AQUA1* plants than in wt plants, mostly in control conditions (Online Resource Table S1). Indeed, the WUE*i* was higher in the transgenic line than in the wt plants in control conditions but this difference was lost under Zn excess (Table 1). Leaf and stem RWC were similar in both lines in CPs and TPs (Table 1). Stem Ψ_w did not show differences between lines and treatments while leaf Ψ_w increased in wt plants under Zn excess (on average + 0.3 MPa, *t*-test $P < 0.05$).

3.2. Mineral element concentrations

Zn concentration increased in all plant compartments of both lines under Zn excess (Fig. 1). It was on average about 20-folds higher in roots and 10-folds higher in leaves. The only differences in Zn content between wt and *AQUA1* TPs was found in the xylem sap in which the heavy metal content was higher in *AQUA1* TPs. The Zn translocation factor (Fig. 2A) was higher in *AQUA1* TPs than in wt TPs (*t*-test $P < 0.05$). The Zn distribution in below and above ground biomasses highlighted a

Table 1
Photosystem II parameters and plant water relations in wt and *AQUA1* lines of *P. alba* under control conditions (1 μM Zn) or Zn excess (1 mM Zn). Each value represents the mean of four biological replicates ± SD. Two-way ANOVA *P*-values and Fisher's LSD test results are reported in the table (Zn, zinc; L, line; ns, not significant; *, $P < 0.05$; **, $P < 0.01$).

Parameters	Control		1 mM Zn		ANOVA		
	wt	*AQUA1*	wt	*AQUA1*	Zn	L	Zn × L
F_v/F_m	0.80 ± 0.017 *b*	0.83 ± 0.001 *a*	0.83 ± 0.001*a*	0.82 ± 0.010 *ab*	ns	ns	*
NPQ	0.12 ± 0.034	0.11 ± 0.042	0.07 ± 0.042	0.09 ± 0.014	ns	ns	ns
ETR	117.8 ± 1.84	115.0 ± 6.26	123.3 ± 6.26	119.8 ± 4.32	ns	ns	ns
WUE*i*	0.87 ± 0.077 *b*	1.59 ± 0.502 *a*	0.92 ± 0.210 *b*	0.58 ± 0.225 *b*	*	ns	**
Leaf RWC (%)	88.4 ± 3.30	91.7 ± 2.86	92.9 ± 2.24	91.6 ± 1.55	ns	ns	ns
Stem RWC (%)	65.8 ± 4.31	69.2 ± 2.95	67.5 ± 4.24	64.7 ± 2.83	ns	ns	ns
Leaf Ψ_w (MPa)	−0.93 ± 0.133	−0.73 ± 0.221	−0.64 ± 0.144	−0.71 ± 0.141	ns	ns	ns
Stem Ψ_w (MPa)	−0.67 ± 0.141	−0.64 ± 0.108	−0.67 ± 0.129	−0.64 ± 0.106	ns	ns	ns

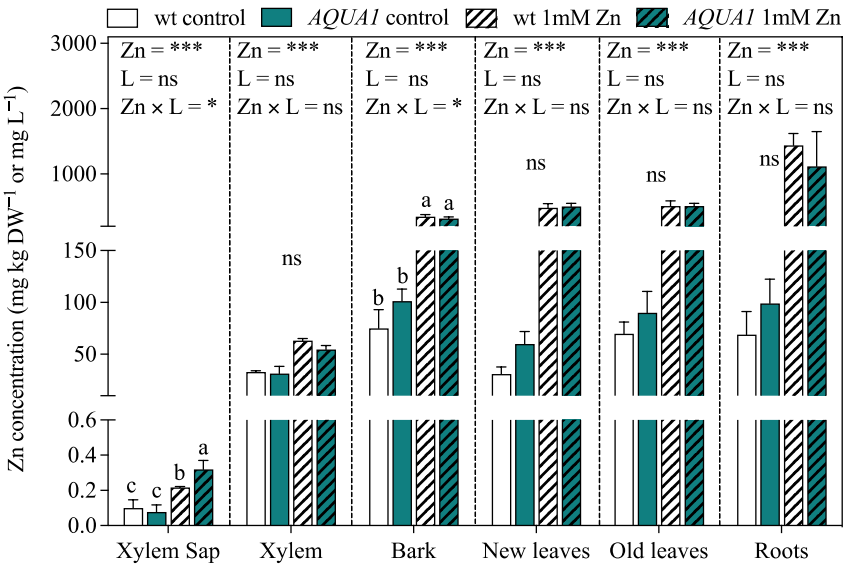

Fig. 1. Zn concentration in xylem sap (mg L^{-1}), xylem, bark, new leaves, old leaves, and roots (mg kg DW^{-1}) in wt and *AQUA1* lines of *P. alba* under control conditions (1 μM Zn) or Zn excess (1 mM Zn). Bars represent the mean of four biological replicates + SD. Two-way ANOVA *P*-values and Fisher's LSD *post-hoc* test results are reported in the figure (Zn, zinc; L, line; ns, not significant; *, $P < 0.05$; ***, $P < 0.001$).

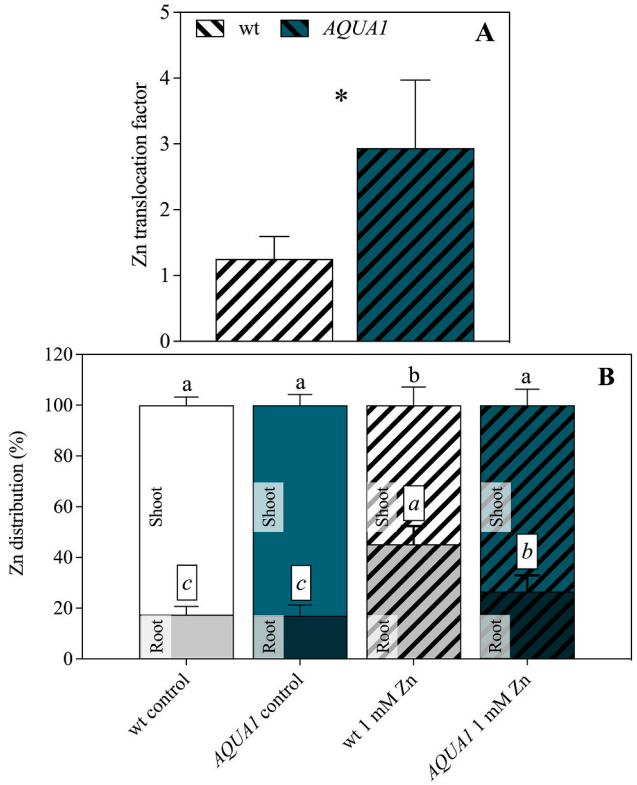

Fig. 2. Zn translocation factor expressed on Zn content (A) and Zn distribution in roots, on the bottom of the bars, and shoots, on the top of the bars, (B) in wt and *AQUA1* lines of *P. alba* under control conditions (1 μM Zn) or Zn excess (1 mM Zn). Bars represent the mean of four biological replicates + SD. *t*-test result is reported in panel A (*, $P < 0.05$). Fisher's LSD *post-hoc* results are reported in panel B in italic font for root and normal font for shoot (Two-way ANOVA Zn × Line $P < 0.01$ for both root and shoot distribution).

higher distribution of Zn in aerial parts (on average 82%) in both lines of CPs (Fig. 2B). Under Zn excess, this distribution was partially relocated in roots, mostly in wt plants.

The main differences in the other mineral elements were retrieved in the xylem sap (Fig. 3). Mn concentration increased in the xylem saps of both lines under Zn treatment while the K concentration decreased. On the contrary, Fe and Ca were not influenced by line and treatment.

Overall, Mn concentration increased in all plant compartments following the Zn trend (Fig. 4A). Within the xylem, Mn concentrations, was lower in *AQUA1* TPs than in wt TPs, as also reported for Zn (*t*-test $P < 0.05$, Fig. 1). K and Fe concentrations decreased within the xylem of both TP lines (Fig. 4B and C) and Fe was also lower in roots of *AQUA1* TPs compared to the control conditions (Fig. 4C).

3.3. Soluble sugar analyses and osmolyte concentration

Osmolytes and soluble sugars were measured in NL, xylem, bark, and xylem sap (Table 2). Zn treatment caused a decrease in xylem sap Ψ_π in both lines. Xylem osmolyte concentration was higher in both lines under Zn excess compared to the wt CPs. Bark and leaf osmolytes did not show differences.

Soluble sugars were considered as the sum of the most representative sugars found in all tissues, *i.e.*, sucrose, glucose, and fructose. Under Zn excess, *AQUA1* plants increased the soluble sugar concentration within the bark. On the contrary, sugar concentration did not change in leaves, xylem, and xylem sap.

The contribution of soluble sugars to the Ψ_π was also calculated in NL, xylem, bark, and xylem sap (Table 2). This parameter was influenced by the Zn treatment and line in leaves and, specifically, it was less negative in *AQUA1* TPs under Zn excess while it did not change in the wt plants. Moreover, the contribution of soluble sugars to the Ψ_π in xylem was lower in *AQUA1* plants than in the wt line. Since the Ca and K concentrations measured in xylem sap corresponded to the free cations, it was also possible to calculate the contribution of these two mineral elements to the xylem sap Ψ_π. This parameter was less negative in both lines under Zn excess.

3.4. Correlation matrixes

The correlation matrixes among Zn, Mn, and Fe within different plant organs and tissues revealed a different trend between wt and *AQUA1* plants (Fig. 5). A main point of diversification was related to root Fe concentration. Specifically, the root Fe concentration was

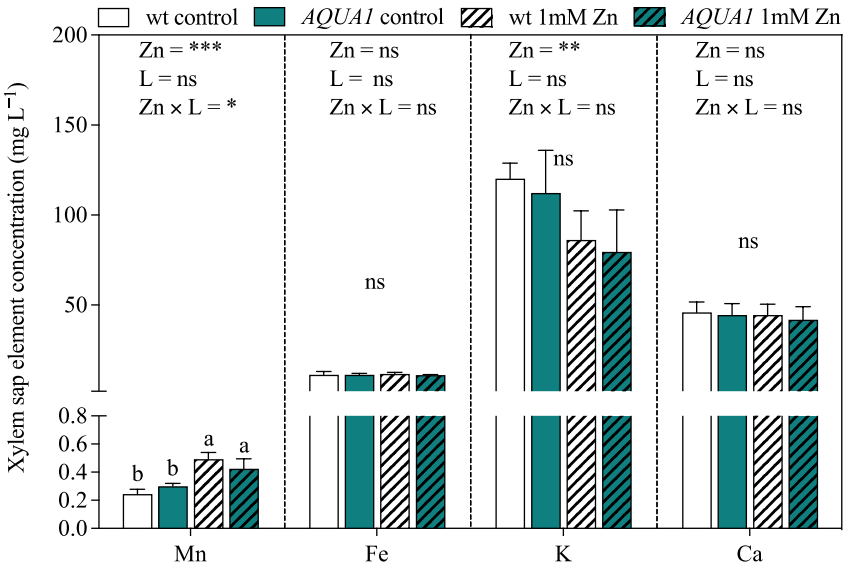

Fig. 3. Mn, Fe, K, and Ca concentrations in xylem sap of wt and *AQUA1* lines of *P. alba* under control conditions (1 μM Zn) or Zn excess (1 mM Zn). Bars represent the mean of four biological replicates + SD. Two-way ANOVA *P*-values and Fisher's LSD test results are reported in the figure (Zn, zinc; L, line; ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

negatively correlated to the Zn concentration measured in all the other plant tissues in the *AQUA1* line (P = * xylem, OL and xylem sap, P = ** bark and NL); on the contrary, a positive correlation was found in wt plants between root Fe concentration and xylem Zn concentration (P = *). Results therefore supported a relation between the decrease of root Fe concentration and the increase of Zn in *AQUA1* line. On the contrary, the xylem Fe concentration was significantly negatively correlated with Zn concentrations of leaf, bark, and xylem in wt plants (P = *) highlighting a different effect of Zn excess on Fe concentration in the two lines. Positive correlations among Zn and Mn were found in both wt and *AQUA1* lines; in particular, Zn concentrations correlated with Mn concentration in NL in both lines, and mostly with the xylem Mn in the wt line and the bark Mn in the *AQUA1* line. A weaker correlation between root Mn and root Zn concentrations was also found in *AQUA1* line compared to the wt plants. Less differences between the two lines were highlighted regarding Mn and Fe correlations. In the *AQUA1* line, the bark Fe concentration was negatively correlated with the leaf Fe concentrations.

A positive correlation was found between the Zn concentration in roots, leaves, bark, and xylem sap and the leaf osmolyte concentration in wt plants (Fig. 6). A negative correlation was found between Zn concentrations in all plant compartments and the bark osmolytes in *AQUA1* plants (P = * in root, xylem, bark, OL and NL, P = ** in xylem sap), as well as between xylem Zn and bark osmolytes in wt plants (P = *). Soluble sugar contribution to the osmotic potential ($\Psi_{\pi(SS)}$) in bark and leaves was found correlated to $\Psi_{\pi(SS)}$ in xylem and bark, respectively, in wt plants.

4. Discussion

Aquaporins are involved in plant transpiration through their contribution to the mechanisms of leaf cooling, gas exchange, xylem mediated water flow, and the transport of nutrients toward the leaves (Grondin et al., 2015; Taiz and Zeiger, 1991). Although several authors have studied the effect of AQP overexpression on plant-water relations and photosynthesis (Kawase et al., 2013; Sade et al., 2010; Uehlein et al., 2003) as well as under heavy metal stress (Vats et al., 2021), less is known about their role in response of osmotic perturbations caused by heavy metal excess.

Our results confirmed the tolerance of *P. alba* clone 'Villafranca' to high Zn concentration considering both the wt and the *AQUA1* overexpressing line. The Zn concentration resulted even higher than the values reported by Romeo et al. (2014) for the wt plants and Ariani et al. (2016) for both lines, respectively. Specifically, Zn concentration was about twofold higher in the wt TPs and three times higher in the *AQUA1* TPs than in the previous experimental trials, probably due to the longer treatment applied in the current experiment (5 weeks longer) as well as the larger plant size. Results confirmed the root as the main organ of Zn accumulation in poplar (Romeo et al., 2014). The Zn translocation factor as well as the Zn distribution in below and above ground biomasses highlighted an improved capacity of *AQUA1* plants to translocate the Zn excess toward the aerial part in comparison to the wt plants. The uptake and translocation of heavy metals to the aerial part is a highly desirable phenotype for the phytoremediation approaches to achieve an efficient pollutant sequestration, constituting a target for the genetic engineering of hyperaccumulating plants (Luo et al., 2016). Ariani et al. (2019) demonstrated in Arabidopsis protoplasts that Zn excess causes a downregulation of poplar *AQUA1* and its re-localization in the membranes of new forming pro-vacuoles supporting the activation of a defence mechanism for reducing water flow. These authors speculated about the possibility to relate the reduction in water flow to a decrease in plant growth and the activation of osmotic potential adjustments, as supported by the downregulation of this gene found by Di Baccio et al. (2011) in *P.* × *euramericana* 'I-214' clone under Zn excess. The constitutive overexpression of *AQUA1* in poplar could potentially prevent this defence mechanism. Indeed, the *AQUA1* plants did not show any decrease of biomass accumulation or stem and leaf water content compared to the wt plants. Although several authors reported a metal induced dehydration (Rucińska-Sobkowiak, 2016), leaf and stem RWC were not impaired by Zn excess in our experimental conditions. Indeed, Fernàndez-Martínez et al. (2014), in poplar in hydroponics, and González et al. (2017), in wheat and barley in soil, reported a stable leaf RWC up to 1 mM or 3,000 ppm of Zn, respectively. Since the heavy metals absorbed by roots are translocated to the aerial parts through their loading into the xylem sap (Luo et al., 2016), the higher Zn concentration retrieved in *AQUA1* xylem sap further supports a higher capacity of this line to drive Zn toward the aerial part through the water flow, even if this does not involve a higher leaf Zn concentration. The equal concentrations of Zn in the aerial parts between the two lines could be due to the young age of plants since the heavy metal

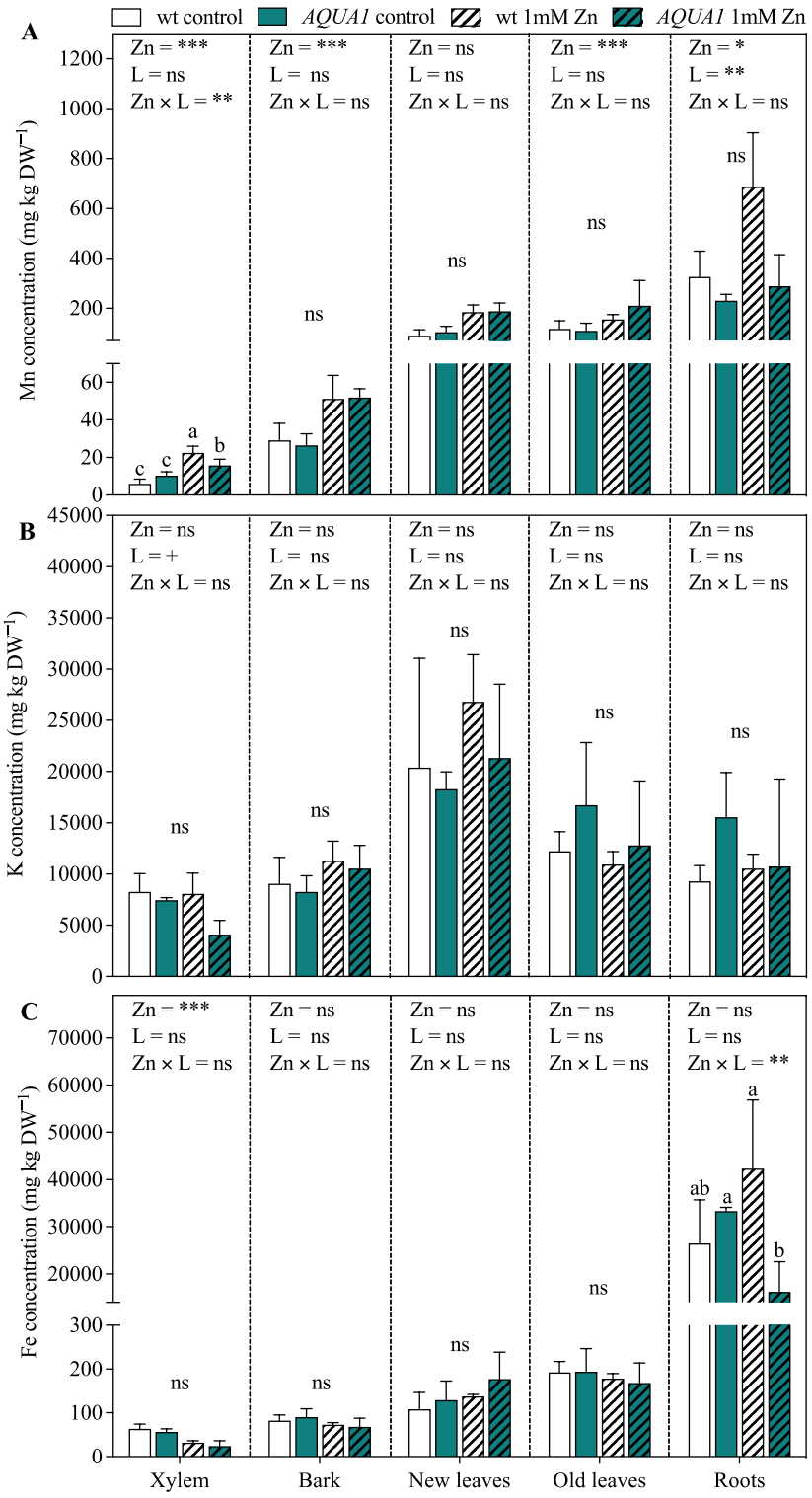

Fig. 4. Mn (A), K (B), and Fe (C) concentrations in xylem, bark, new leaves, old leaves, and roots in wt and *AQUA1* lines of *P. alba* under control conditions (1 μM Zn) or Zn excess (1 mM Zn). Bars represent the mean of four biological replicates + SD. Two-way ANOVA *P*-values and Fisher's LSD test results are reported in the figure (Zn, zinc; L, line; ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

accumulation depends on the plant biomass (Patra et al., 2020). Thus, a future perspective might be to test the transgenic line for longer periods.

Results further confirmed in control conditions the decrease in g_s and E in *AQUA1* plants already reported by Ariani et al. (2016) highlighting a higher WUE*i* of *AQUA1* line in control conditions. The same authors described under Zn treatment a decrease in g_s and A in both wt and *AQUA1* plants, while the Zn treatment did not affect these parameters in our experimental conditions even if the WUE*i* decreased in *AQUA1* line. The absence of decrease of these parameters in comparison with Ariani et al. (2016) might be due to the different duration of Zn treatment in

Table 2
Osmolyte and soluble sugars concentrations and soluble sugar contribution to the osmotic potential in wt and *AQUA1* lines of *P. alba* under control conditions (1 μM Zn) or Zn excess (1 mM Zn). Each value represents the mean of four biological replicates ± SD. Two-way ANOVA *P*-values and Fisher's LSD test results are reported in the table (Zn, zinc; L, line; ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Ψ_π = osmotic potential, Osm = osmolytes, SS = soluble sugars.

Parameters	Control		1 mM Zn		ANOVA		
	wt	*AQUA1*	wt	*AQUA1*	Zn	L	Zn × L
Xylem sap Ψ_π (MPa)	−0.05 ± 0.013	−0.05 ± 0.010	−0.04 ± 0.005	−0.03 ± 0.009	**	ns	ns
Xylem Osm (mOsm g^{-1} DW)	0.45 ± 0.073 *b*	0.58 ± 0.047 *ab*	0.71 ± 0.056 *a*	0.61 ± 0.071 *a*	***	ns	**
Bark Osm (mOsm g^{-1} DW)	1.77 ± 0.296	2.12 ± 0.960	1.84 ± 0.185	1.96 ± 0.347	ns	ns	ns
Leaf Osm (mOsm g^{-1} DW)	1.76 ± 0.401	1.80 ± 0.186	1.65 ± 0.218	2.06 ± 0.228	ns	ns	ns
Xylem sap SS (μg mL^{-1})	159.3 ± 73.31	156.6 ± 55.57	156.6 ± 60.78	194.9 ± 33.05	ns	ns	ns
Xylem SS (mg g^{-1} DW)	14.5 ± 3.50	9.4 ± 2.69	13.6 ± 4.18	16.4 ± 3.25	ns	ns	ns
Bark SS (mg g^{-1} DW)	66.3 ± 9.22 *ab*	48.2 ± 9.65 *b*	56.7 ± 12.49 *ab*	72.2 ± 15.35 *a*	ns	ns	*
Leaf SS (mg g^{-1} DW)	69.2 ± 1.31	61.7 ± 11.64	65.7 ± 11.88	61.8 ± 12.49	ns	ns	ns
Xylem sap $\Psi_{\pi(SS)}$ (kPa)	−1.7 ± 0.83	−1.8 ± 0.84	−1.8 ± 0.62	−2.7 ± 0.92	ns	ns	ns
Xylem sap $\Psi_{\pi(ME)}$ (kPa)	−9.7 ± 1.80	−12.0 ± 4.87	−8.2 ± 1.49	−7.6 ± 1.90	*	ns	ns
Xylem $\Psi_{\pi(SS)}$ (MPa)	−0.53 ± 0.112	−0.47 ± 0.016	−0.51 ± 0.128	−0.26 ± 0.088	ns	*	ns
Bark $\Psi_{\pi(SS)}$ (MPa)	−0.57 ± 0.163	−0.52 ± 0.099	−0.72 ± 0.121	−0.67 ± 0.142	ns	ns	ns
Leaf $\Psi_{\pi(SS)}$ (MPa)	−0.68 ± 0.100 *b*	−1.16 ± 0.058 *a*	−0.68 ± 0.079 *b*	−0.51 ± 0.082 *b*	***	**	***

this trial suggesting that an acclimation occurred during a prolonged Zn excess. Indeed, in literature contrasting results were reported in poplar for gas exchange parameters under Zn treatment. As example, Durand et al. (2010) reported a stable stomatal conductance under Zn treatment in *P. tremula* × *P. alba* cultivated in soil. On the contrary, other authors tested Zn excess in *P.* × *euramericana* 'I-214' clone and in *P. deltoides* × *maximowiczii* 'Eridano' clone reporting a decrease in g_s or in A (Fernàndez-Martínez et al., 2014).

The Mn, K, and Fe concentrations were overall not altered by the genetic modification in all compartments of the transgenic plants in comparison with wt plants while they were modified by Zn excess in both lines, particularly in xylem. The Zn increase coupled with a higher Mn concentration in both wt and *AQUA1* plants. Indeed, the association between Zn and Mn transport due to the low specificity of metal transporters has been well documented (Socha and Guerinot, 2014). The Fe concentration was lower in the xylem of both lines while this element decreased only in root of *AQUA1* plants. The Fe decrease could be explained by the competition of this element with Zn for the citrate chelation for transport (Cornu et al., 2015). The strong link between these elements has been highlighted in Fe deficiency experiment in which plant roots have shown to maintain the Fe homeostasis indirectly increasing Mn and Zn concentration in plant tissues (Chen et al., 2019) even to the point of causing Zn stress (Kanai et al., 2009). Thus, a possible explanation of the higher Zn uptake capacity of *AQUA1* line might be related to a higher specificity of this line for Zn than for Fe, as highlighted by the Fe decrease in TP roots and the negative correlations between these two elements found in *AQUA1* root. Indeed, different responses among heavy metal transporters between wt and *AQUA1* lines have been already highlighted at gene transcription level under Cd excess (Neri et al., 2020). Moreover, a different contribution of symplastic and apoplastic transport cannot be also excluded in the transgenic line since the two types of transport are both involved in heavy metal uptake (Rucińska-Sobkowiak, 2016).

The increase in Zn concentration in xylem sap coupled with a decrease in K concentration in both TP lines, followed also by a decrease in xylem K, probably linked with the attempt of maintaining a stable sap osmotic potential under Zn excess. Indeed, the role of Zn in osmotic adjustments has been already showed in sunflower under drought stress (Jan et al., 2022). Moreover, it has been shown in poplar an increase in K efflux from the roots under heavy metal treatment (Palm et al., 2017) that might explain the decrease of free K found in xylem vascular flow in our experimental conditions. Since K is a key molecule in osmotic adjustments (Blum, 2017) as expected the xylem sap Ψ_π was also higher in TPs compared to CPs (i.e., lower osmolytes). Beyond the less negative values in xylem sap Ψ_π in both lines under Zn excess, xylem osmolytes increased only in wt TPs. This result might highlight a higher sensitivity of wt plants to the Zn excess that it is known to trigger the activation of plant defence mechanisms including the accumulation of osmotically active molecules (Kaur and Garg, 2021). This process is further confirmed by the significant positive correlations found between Zn concentrations and leaf osmolytes in wt plants. However, it must be highlighted the active osmotic process in act in wt line was not related with K or soluble sugar fluctuations. On the contrary, *AQUA1* plants showed a higher concentration of soluble sugars in the bark under Zn excess. An increase of soluble sugars in *P. alba* bark has been already found under abiotic stress as water limited conditions (Traversari et al., 2020) and could be due to a higher photosynthetic activity of bark that confer increased drought resistance (Bloemen et al., 2016) and therefore in our experimental conditions it could be triggered by the osmotic perturbations related to the Zn excess. However, a more than halved leaf $\Psi_{\pi(SS)}$ was found in *AQUA1* plants under Zn excess supporting also a sugar flow from the leaves to the bark that did not affect the overall concentration of leaf soluble sugars but potentially triggered an increase of bark soluble sugars.

In conclusion, the *AQUA1* overexpression confirmed to enhance *P. alba* 'Villafranca' capacity to remediate a Zn contamination also after a long-term exposure. The transgenic line highlighted a preferential translocation of Zn toward the leaves, a phenotype highly desired for phytoremediation approaches. A possible explanation of this phenotype could be related to a more specific selectivity for Zn than for other competing elements at root level, such as Fe, as well as to an improved tolerance to xylem osmotic perturbations triggered by the Zn accumulation. This different behaviour could be related to the increased WUE*i* in control conditions and the increase in bark soluble sugars under Zn excess that may lead to a higher tolerance to water and osmotic perturbations decreasing the water loss as highlighted by the lower alteration in the osmolytes found in comparison with the wt line.

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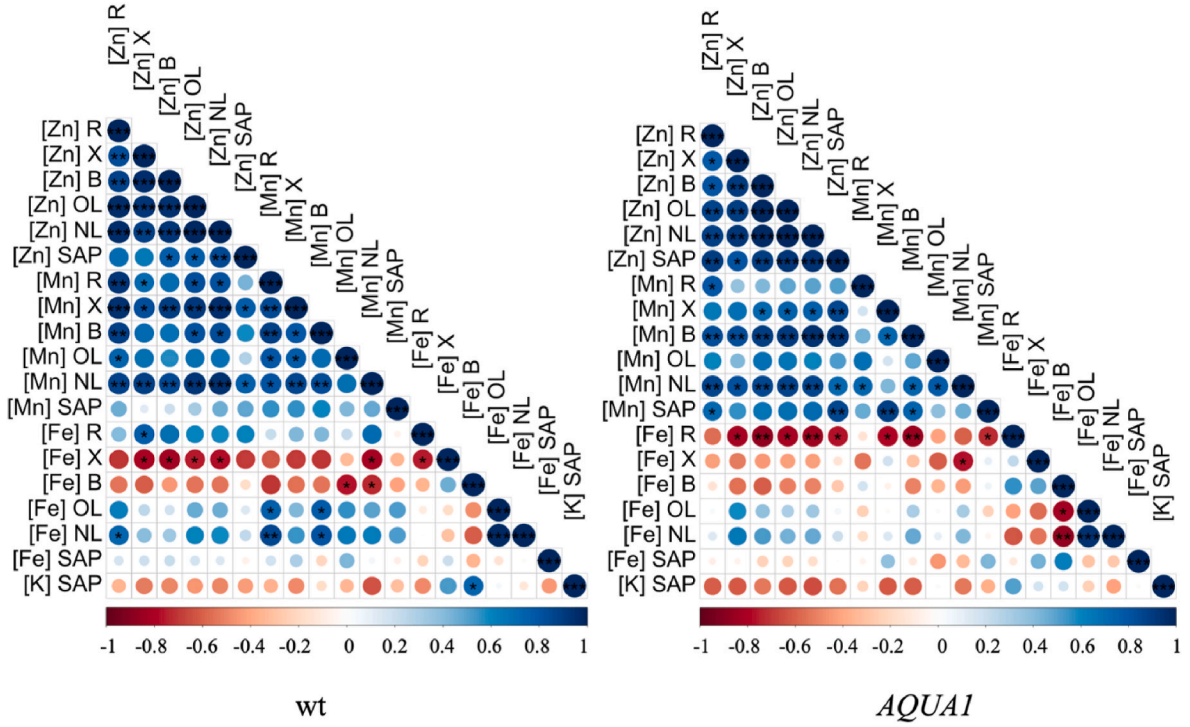

Fig. 5. Pearson correlation matrixes among Zn, Mn, and Fe concentrations in wt and *AQUA1* lines of *P. alba*. R = roots, X = xylem, B = bark, OL = old leaves, NL = new leaves, SAP = xylem sap.

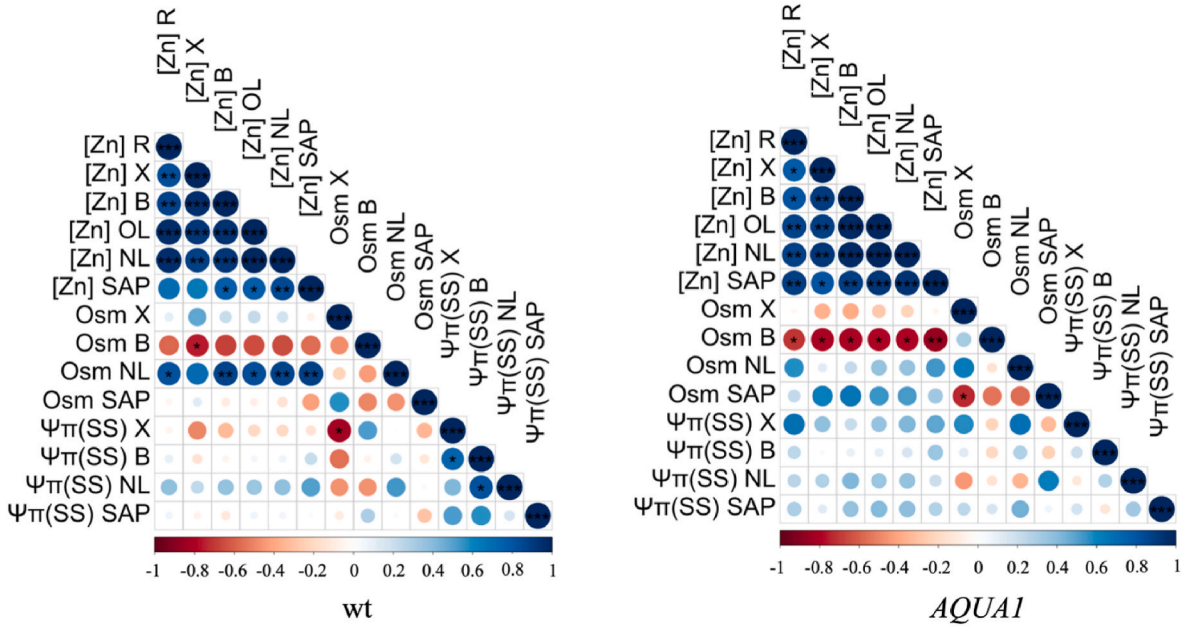

Fig. 6. Pearson correlation matrixes among Zn concentration, osmolytes (Osm), and soluble sugar contribution to the osmotic potential ($\Psi_{\pi(SS)}$) in wt and *AQUA1* lines of *P. alba*. R = roots, X = xylem, B = bark, OL = old leaves, NL = new leaves, SAP = xylem sap.

CRediT authorship contribution statement

Andrea Neri: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Alessandra Francini:** Methodology, Supervision, Writing – review & editing. **Alessio Giovannelli:** Methodology, Resources, Supervision, Writing – review & editing. **Silvia Traversari:** Data curation, Formal analysis, Methodology, Writing – original draft. **Luca Sebastiani:** Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2024.108528.

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