

Over-expression of AQUA1 in *Populus alba* Villafranca clone increases relative growth rate and water use efficiency, under Zn excess condition

Andrea Ariani^{1,2} · Alessandra Francini¹ · Andrea Andreucci³ · Luca Sebastiani¹

Abstract

Key message Transgenic *Populus alba* over-expressing a TIP aquaporin (*aqual*) showed a higher growth rate under Zn excess, suggesting that *aqual* could be involved in water homeostasis, rather than in Zn homeostasis. **Abstract** *Populus* is the internationally accepted model for physiological and developmental studies of tree traits under stress. In plants, aquaporins facilitate and regulate the diffusion of water, however, few poplar aquaporins have been characterized to date. In this study, we reported for the first time an in vivo characterization of *Populus alba* clone Villafranca transgenic plants over-expressing a TIP aquaporin (*aqual*) of *P. x euramericana* clone I-214. An AQUA1:GFP chimeric construct, over-expressed in *P. alba* Villafranca clones, shows a cytoplasmic localization in roots, and it localizes in guard cells in leaves. When over-expressed in transgenic plants, *aqual* confers a higher growth rate compared to wild-type (wt) plants, without affecting chlorophyll accumulation, relative water content (RWC), and fluorescence performances, but increasing the intrinsic Transpiration Efficiency. In response to Zn (1 mM), transgenic lines did not show a significant increase in Zn accumulation as compared to wt plants, even though the over-expression of this gene confers higher tolerance in root tissues. These results suggest that, in poplar plants, this gene could be principally involved in regulation of water homeostasis and biomass production, rather than in Zn homeostasis.

Keywords Aquaporin · Villafranca clone · Growth · Photosynthesis · Zinc

Water is the medium of life, and almost every living organisms need this molecule for accomplishing its basic physiological functions. Before the discovery of the first water channel protein in red blood human cells (Agree et al. 1987), it was thought that water, being a small polar molecule, crosses biological membrane only through passive diffusion across cellular lipid bilayer (Maurel 1997). After the discovery of the first human aquaporin, novel perspectives were

Introduction

opened to the study of plant–water relationships. Aquaporins facilitate and regulate the diffusion of water and other small molecules (e.g., urea, ammonium and glycerol) across biological membranes and are involved in almost all plant physiological processes such as growth, development, and response to biotic and abiotic stresses (Li et al. 2013). Aquaporins belong to the major intrinsic protein superfamily (MIP), and in plants form a large gene family with more than thirty members identified in each genome of *Arabidopsis*, *Zea mays*, *Oryza sativa*, *Populus trichocarpa* and *Phaseolus vulgaris* (Quigley et al. 2001; Chaumont et al. 2001; Sakurai et al. 2005; Gupta and Sankararamkrishnan 2009; Ariani and Gepts 2015). In higher plants, these proteins can be divided into 5 sub-families, according to sequence similarity and sub-cellular localization: the Plasma membrane Intrinsic Proteins (PIPs), Tonoplast Intrinsic Proteins (TIPs), Nodulin26-like Intrinsic Proteins (NIPs), Small basic Intrinsic Proteins (SIPs), and unrecognized X Intrinsic Proteins (XIPs) (Li et al. 2013).

Populus spp. is the internationally accepted model for physiological and developmental studies on tree-specific traits, that are not possible in herbaceous plants (Tuskan et al. 2006; Wullschlegel et al. 2012). Due to the high biomass production, fast growth rate, and transpiration of this species, aquaporins are probably central in the regulation of water flow and in the developmental processes of these plants. In addition, across sequenced plants, *Populus trichocarpa* has the highest number of MIP genes identified in its genome sequence to date (Gupta and Sankararamkrishnan 2009) probably as a consequence of the Salicoid genome duplication (Rodgers-Melnick et al. 2012). Despite the high number of aquaporin genes in the *Populus* genome, their physiological roles have been only partially elucidated in this species, mainly through expression analysis. In particular, in different *Populus* genotypes aquaporins are differentially expressed during root development, mycorrhizal symbiosis, and in recovery to xylem embolism (Kohler et al. 2003; Marjanovic et al. 2005; Secchi and Zwieniecki 2010). Furthermore, some aquaporin genes were found to be responsive to abiotic stresses and hormone treatments (Bae et al. 2011; Hacke et al. 2010; Lopez et al. 2012). A more comprehensive survey of aquaporin expression profiles of several *Populus* microarray experiments highlighted a high degree of functional divergence across duplicated genes of this family, suggesting then specific regulation of single aquaporin genes in response to environmental cues (Cohen et al. 2013).

Even though gene expression analysis could give valuable information on aquaporin function in plant physiology (Sakurai et al. 2005; Besse et al. 2011), *Agrobacterium*-mediated transformation seems to be a better approach for this kind of study, allowing in vivo functional characterization of single gene products and analysis of their role at whole plant level. This technology has been widely exploited for studying aquaporin genes in several herbaceous plant species such as *Arabidopsis thaliana* (Cui et al. 2008; Postaire et al. 2010; Lee et al. 2012), *Oryza sativa* (Li et al. 2008; Matsumoto et al. 2009), *Solanum lycopersicum* (Sade et al. 2009), and *Vitis vinifera* (Perrone et al. 2012). These studies have allowed a better characterization of the roles of single aquaporin genes in plant–water relations, biomass production, and stress responses. Interestingly, studies on stress responses in transgenic plants over-expressing an aquaporin of interest highlighted opposite effect for different aquaporins, that could confer either higher tolerance or sensitivity (Li et al. 2013). Thanks to the availability of several protocols for *Agrobacterium*-mediated transformation of different *Populus* clones (Song et al. 2006), several reverse engineering approaches have been applied for studying different *Populus* genes such as transcription factors involved in secondary growth differentiation (Robinschon et al. 2011; Du et al. 2011) and stress responses (Su et al. 2011). However, few studies using *Agrobacterium*-mediated transformation of endogenous aquaporins have been performed in this species to date.

In this study, we report an analysis of *P. alba* (Vil-lafranca clone) transgenic lines over-expressing an aquaporin (GenBank: GQ918138) of *P. x euramericana* I-214 clone in different expression vectors. Transgenic lines carrying an AQUA1:GFP construct under the 35S promoter have been created for analyzing the sub-cellular localization of this aquaporin in different organs and tissues. Additionally, AQUA1:HA over-expressing lines have been characterized for growth and photosynthetic parameters, in comparison to wild-type (wt) plants, in order to understand the physiological role of this protein in *Populus*.

Response to excess Zn is a complex phenomenon in poplar trees, entailing variation in biochemical and physiological processes, but also anatomical adaptation to toxic concentration of this metal (Di Baccio et al. 2009, 2011). These differences were related also to organ-specific variation in transcriptome and proteome, with activation of ROS detoxifying mechanisms, regulation of heavy metal transporters, and Nitrogen and Carbon metabolisms in response to excess Zn (Di Baccio et al. 2011; Romeo et al. 2014a; Ariani et al. 2015). Physiological analysis of

response to excess Zn in different poplar clones showed also a genotype specific response toward this metal, with variation in sensitivity, metal concentration, and allocation among different poplar clones (Romeo et al. 2014b). However, a common response was the concentration of the majority of Zn in roots tissues, probably for protecting photosynthetic tissues.

Previous studies on *aqual* showed its down-regulation in both leaves and roots in response to excess Zn in I-214 hybrid poplar clone, regulation of sub-cellular localization by post-translational modifications in response to this heavy metal in Arabidopsis protoplast, and also a protective role of this gene in yeast Zn-hypersensitive strains (Di Baccio et al. 2011; Andreucci et al. 2015). Several studies highlighted the ability of some aquaporins to transport metals and to confer heavy metal resistance when over-expressed in transgenic plants (Zhang et al. 2008; Hove and Bhavane 2011; Negishi et al. 2012). In addition, *P. alba* (Villafranca clone) has been reported as a promising poplar clone for possible phytoremediation applications (Romeo et al. 2014b).

This experiment was set up in order to test the hypothesis that *aqual* transgenic lines have different Zn tolerance in comparison to the wt.

Material and method

Plant transformation and in vitro propagation

The coding sequence of *aqual* was excised from pYES vector with XbaI and BamHI restriction enzymes. The XbaI end was filled in for having a blunt-end of the excised *aqual* coding fragment. The pENTR 1A entry vector was digested with BamHI and EcoRV restriction enzymes and the *aqual* coding fragment was ligated inside this vector. The *aqual* full coding DNA sequence (CDS) inside pENTR 1A vector was then cloned in different expression vectors using Gateway (LifeTechnology) recombination technology according to manufacturer's instruction (Zhang et al. 2006; Nakagawa et al. 2008). Two different expression vectors were used for the different purpose of this study. The first vector was the pMDC45 that determines the expression of AQUA1:GFP chimeric construct under the 35S promoter and was used for subsequent sub-cellular and organs localization of AQUA1:GFP protein in transgenic plants. The second vector, in which *aqual* sequence was cloned, was the pEarlyGate-201 that carried the full CDS sequence of *aqual* in frame with the hemoagglutinin (HA) tag. Plants transformed with this vector were used for subsequent phenotypic characterization of AQUA1 over-expressing transgenic poplars, since the HA-tag should not disturb *aqual* functions and folding.

Both vectors were transformed in the *Agrobacterium* LB4404 strain (LifeTechnology) with the Gene Pulser X-cell electroporation system (Biorad, Berkeley, CA, USA) according to manufacturer's protocol, and in vitro plantlets of *Populus alba* Villafranca L. clone were transformed with this *Agrobacterium* strain as described in Confalonieri et al. (2000). After regeneration, transformants were selected in selective media with 10 mg/L hygromycin or 5 mg/L BASTA, depending on the transformation vectors utilized. Plants, able to root in these media (i.e., resistant to the selection), were considered transgenic and further propagated and multiplied in WPM medium as separated lines.

Expression analysis

Expression analyses of 10 separate transgenic lines (data not shown) were performed first on in vitro whole plantlets using three technical replicates for each line following the same methodology reported below for in vivo plants. Then, two transgenic lines were selected for acclimation and in vivo analysis. After, we will refer to the two transgenic lines as line 16 and line 22. For in vivo experiments of *aqual*:HA over-expressing lines, the maintenance of the transgene was tested by performing the last in vitro multiplication step (before acclimation) on selective media. In addition, the in vitro culture of a group of wt *Populus alba* L. Villafranca clone was synchronized with transgenic lines during propagation and multiplication steps and all the plants for in vivo analysis were acclimated together. In vivo expression was then analyzed on leaves of two acclimated transgenic lines (line 16 and 22), performing three technical replicates for three independent biological samples.

The relative expression of *aqual* transcript in AQUA1:HA transgenic lines was compared with the endogenous gene of *P. alba* Villafranca clone. Total mRNA was extracted using 80–100 mg of frozen (-80 °C) leaves, with the RNeasy plant mini kit (Qiagen) according to the manufacturer's protocol. Total RNA was treated with RNase-free DNase (Qiagen) in order to remove genomic DNA contamination prior to quantification and reverse transcription. RNA quality and concentration were evaluated with the Experion RNA StdSens Kit (Biorad), and 1 µg of total RNA was reverse transcribed with Quantitect Reverse Transcription Kit (Qiagen) according to the man-

manufacturer's protocol. Real-time PCR analyses were performed using the Quantitect SYBR Green PCR Kit (Qiagen) according to manufacturer's protocol on ECO Real-Time PCR System (Illumina Inc. San Diego, USA).

As a reference gene, the primers from Brunner et al. (2004) were tested and *alfa-tubulin* (forward 5⁰AGGTTC TGGTTTGGGGTCTT3⁰, reverse 5⁰TGTCACGTCTGTG TGCTCAA3⁰) was selected as for more suitable reference gene for our experimental condition, allowing the gene normalization and quantification. For Real-Time PCR experiments on *aqual* gene, the following primers were used: forward 5⁰CCTCCGTGGCATTCTGTATT3⁰, reverse 5⁰CAAAGGTCCAGCCCAGTAAA3⁰.

Confocal imaging

The fluorescence of in vitro transgenic plants overexpressing the AQUA1-GFP construct were visualized by confocal laser scanning microscopy. Leaves and roots of transgenic plants were analyzed with an inverted Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems, <http://www.leica.com>), using a 63X NA 1.3 Plan-Apo oil-immersion objective at 1024 × 1024 pixel resolution. For GFP fluorescence analyses, we used the 488 nm excitation line of an argon ion laser and the emission light was dispersed and recorded at 500–535 nm.

Plant growth and Zn treatment

Plantlets of *Populus alba* L. Villafranca clone derived from in vitro culture were acclimatized in vivo for 3 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ supplied by fluorescent lights). Plantlets from in vitro culture were transferred from Magenta vessels with WPM half-strength solid medium (Lloyd and McCown 1981) to pots filled with perlite (Laterlite, Milano Italy), closed in plexiglass boxes in order to maintain the high humidity (100 %) typical of in vitro cultures. During the acclimation process, the nutrient supply was gradually changed from WPM half-strength liquid medium to Hoagland's solution (Arnon and Hoagland 1940) and relative humidity was reduced from 100 % to 65–70 % (growth chamber parameter) in order to adapt plants to the in vivo conditions. After the acclimation period, the plants were transferred into plastic pots, containing 8–20 cm^3 expanded Agrileca clay (Laterlite, Milano Italy) and grown hydroponically on a basic Hoagland's nutrient solution at pH 6.2 as described by Arnon and Hoagland (1940), renewed every week and continuously aerated by aquarium pumps (250 l h^{-1}). After 4 weeks of acclimation to hydroponic conditions, five (5) plants were destructively sampled in order to determine the initial fresh (FW) and dry weight (DW) of roots, stem, and leaves. The remaining plants (10 for each transgenic line and 10 for wt) were randomly divided in two groups ($n = 5$) and assigned to two different Zn treatments for 5 weeks (35 days): 1 μM Zn as control and 1 mM Zn as treatment. According to previous knowledge (Ariani et al. 2015; Di Baccio et al. 2011), 1 mM was the Zn concentration in which *aqual* was down-regulated in *P. x euramerica* I-214 clone in both roots and leaves (Di Baccio et al. 2011). In addition, this concentration has also been shown to increase Zn accumulation in *Populus alba* L. Villafranca clone in hydroponic condition (Romeo et al. 2014b). Zn was supplied as zinc nitrate hexahydrate ($\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$), and iron as Fe-tartrate instead of Fe-EDTA for avoiding possible Zn chelation by EDTA.

Leaf weight ratio (LWR), stem weight ratio (SWR), and root weight ratio (RWR) were calculated as the fraction of the total biomass allocated to the leaves, stem, and root [organ mass (g)/mass of plant (g)] to whole plants (Hunt 1978).

Growth and Zn content analysis

At the end of the experiment ($T_1 = 35$ days), each plant was harvested, rinsed with deionized water, and separated into leaf, stem, and root. Disks (1 cm^2) from fully expanded leaves, with leaf plastochron index (LPI) equal to 5, were collected in pre-weighed Petri dishes. The Petri were sealed immediately and weighed to obtain the fresh weight (FW). Leaf samples were placed in distilled water and kept at 4 °C overnight. The samples were then weighed

(saturated weight, SW) and then dried at 60 °C for 2 days

and weighed (dry weight, DW). RWC was calculated from the equation: $\text{RWC} = \frac{\text{FW} - \text{DW}}{\text{SW} - \text{DW}} \times 100$.

DW of each sampled plant parts harvested was determined after drying at 60 °C until their weight remained constant. Relative growth rate (RGR) was determined as the differences between the natural logarithm of DW at T_1 and the natural logarithm of the mean DW at T_0 , divided for the day of treatment (35 days) for different plant parts or for the whole plants. Tolerance indices were calculated as described by Wilkins (1957) using the ratio between the DW biomass of treatment and control conditions. The mineral concentration inside leaves, stems, and roots was detected by Kjeldahl (1883) method (N); atomic absorption (Zn); ICP analysis (Ca, Mg, P, K, Na, S, B, Fe, Mn, Cu). The acid

mineralization was done on 0.5 g dry homogeneous samples. Samples were digested for 10 min into a microwave-digesting machine with a solution composed of 6 ml HNO₃ 65 % and 1 ml H₂O₂ 33 %. The resulting solutions were filtered and diluted with ultra-pure water to a 25 ml volume and then analyzed with atomic absorption or ICP analysis.

Chlorophyll, fluorescence, and gas exchange measurements

Changes in chlorophyll concentration during the leaf maturation process was measured using SPAD meter (SPAD502 Plus Chlorophyll Meter, Spectrum, Plainfield, IL, USA) on three consecutive fully expanded leaves randomly selected on each plant.

Chlorophyll *a* fluorescence transients were determined on dark-adapted leaves (30 min), using a portable Handy PEA (Hansatech, UK). The measurements were taken on the leaf surface (4 mm diameter) exposed to an excitation light intensity (ultrabright red LEDs with a peak at 650 nm) of 3000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (600 W m^{-2}) emitted by three diodes. Leaf fluorescence detection was measured by fast-response PIN photodiode with RG9 long-pass filter (Hansatech, technical manual).

Instantaneous gas exchange and photosynthetic CO₂ response curves (A/Ci) were measured on the fully expanded leaf LPI = 5 of three plants for each Zn treatment and lines. All the measurements were conducted on plants at the end of the Zn treatments with a portable photosynthesis system (CIRAS-1 PP Systems, Hearts, UK) equipped with a Parkinson leaf chamber in saturating light between 1000 and 1200 h at an air temperature of 26–30 °C. Photosynthetic rate (A), stomatal conductance (g_s), and intercellular (Ci) CO₂ were measured. Leaf chamber temperature and humidity were adjusted to maintain a leaf to air vapor pressure difference of 1.1 kPa. The CO₂ response curves were obtained by changing the [CO₂] entering the cuvette from 50 to 1500 $\mu\text{mol mol}^{-1}$ with an external CO₂ cartridge mounted on the CIRAS console and automatically controlled by a CO₂ injector. Gas exchange was determined at each step after exposure of the leaf to the new [CO₂] for 120–240 s; Q was maintained at 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Statistical analysis

The experiment was set up in a completely randomized block design with five replicate plants ($n = 5$) for each treatment.

The *t* test analyses was performed on data ($n = 3$) about in vivo relative expression of *aqual* transcripts in leaves of transgenic plants vs wt plants. Data ($n = 5$) of LMR, SWR, RWR, SPAD, RWC, RGR calculated on different lines after 35 days of 1 IM and 1 mM Zn treatment, Zn concentration in leaves, stems, and roots, CO₂ assimilation at saturating light level, stomatal conductance to water vapor, and the intrinsic Transpiration Efficiency were subjected to a two-way ANOVA for statistical evaluation of the effects produced by Zn, line and interaction of these two factors. Means were subjected to Tukey's Multiple Comparison Test at 0.05 probability level.

Data of RGR ($n = 5$) calculated after 35 days of 1 IM Zn treatment on wt, line 16 and line 22, Tolerance index ($n = 5$) and *aqual* relative expression ($n = 3$) in vivo leaves were subjected to one-way ANOVA and Tukey's Multiple Comparison Test ($P \leq 0.05$). ANOVA was performed using NCSS 2000 Statistical Analysis System Software.

A principal component analysis (PCA) was performed to help the identification of the element distributions in plants in relation to Zn uptake. PCA is designed to reduce a dataset containing a large number of variables to a smaller size by finding a new set of variables called components, each being a linear combination of the original variables. The number of derived components is the same as the number of original variables; in this case, there are 10 element measurements constituting the variables and hence 10 components. When the original set of variables is closely correlated, it is possible to derive a smaller number of underlying components to represent the original dataset while retaining most of the information in it. A score is assigned to each observation with respect to each principal component; this allows the identification of patterns in the data across observational units. PCA analyses was performed on STATISTICA Software.

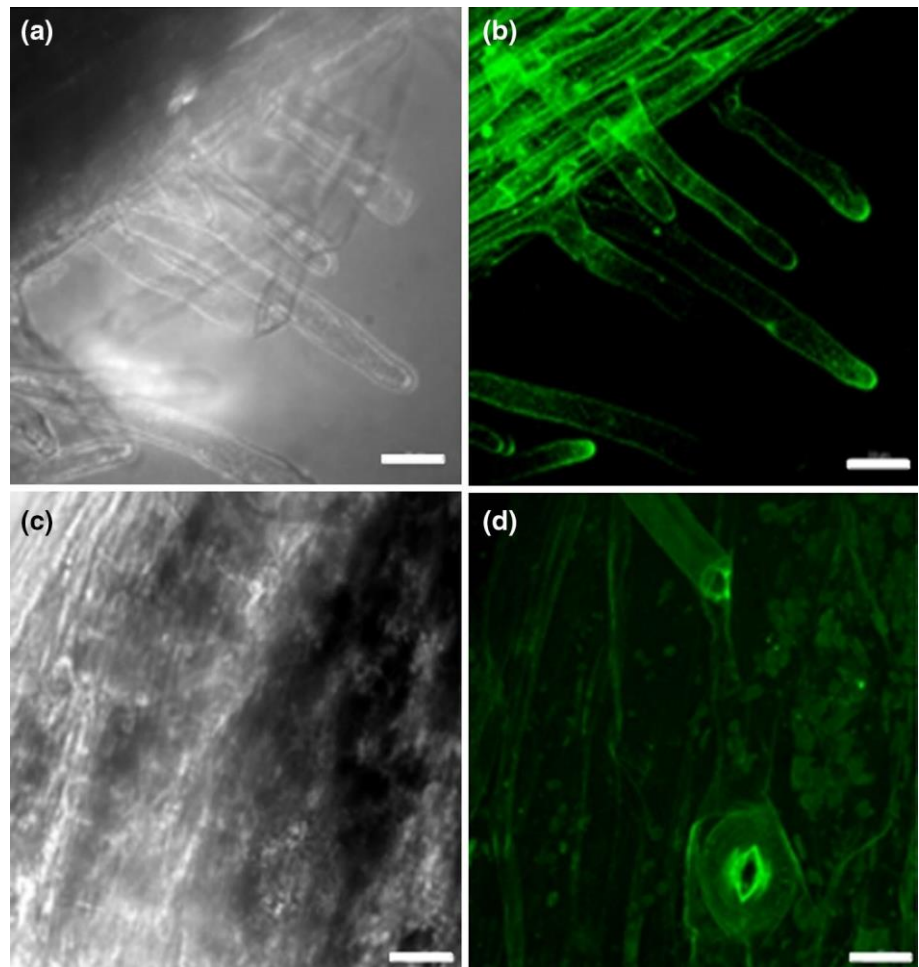
Results

In vitro plant regeneration and AQUA1:GFP localization

At the end of in vitro regeneration, the transformation processes showed an efficiency of 6–7 % for the two

constructs, similar to those observed previously in the transformation protocol of the same clone (Confalonieri et al. 2000). Ten transgenic lines were created with the AQUA1:HA construct (Fig. 1 S1), and 7 with the AQUA1:GFP construct (data not shown). The expression of *aqual:ha* in all transgenic plants was assayed by RT-qPCR by comparing the endogenous expression of *aqual* *P. alba* orthologs. Transgenic in vitro plantlets showed an increase of *aqual* expression in comparison to the endogenous transcript of wt plants (data not shown). When over-expressed in transgenic in vitro plantlets, the fluorescent signal of the AQUA1:GFP was detected in guard cells in leaves; however, in roots of the same plants, the fluorescent signal was detected in the cytoplasm (Fig. 1). From the over-expressing *aqual* in vitro plants, two different transformed lines (line 16 and line 22) were chosen for molecular and physiological analyses. To estimate the levels of *aqual* up-regulation in the acclimated transgenic plants, the qRT-PCR analyses were performed on fully expanded leaves of in vivo plants. *Aqual* gene was over expressed in leaves of transgenic line 16 and line 22 (269 and 25 FC, respectively) under 1 IM and 1 mM Zn treatments (Fig. 2).

Fig. 1 Confocal imaging of AQUA1:GFP in transgenic over-expressing *P. alba* plants. a root, *white field*; b root, 488 nm; c leaf, *white field*; (d) leaf, 488 nm. Bar represent 20 μ m



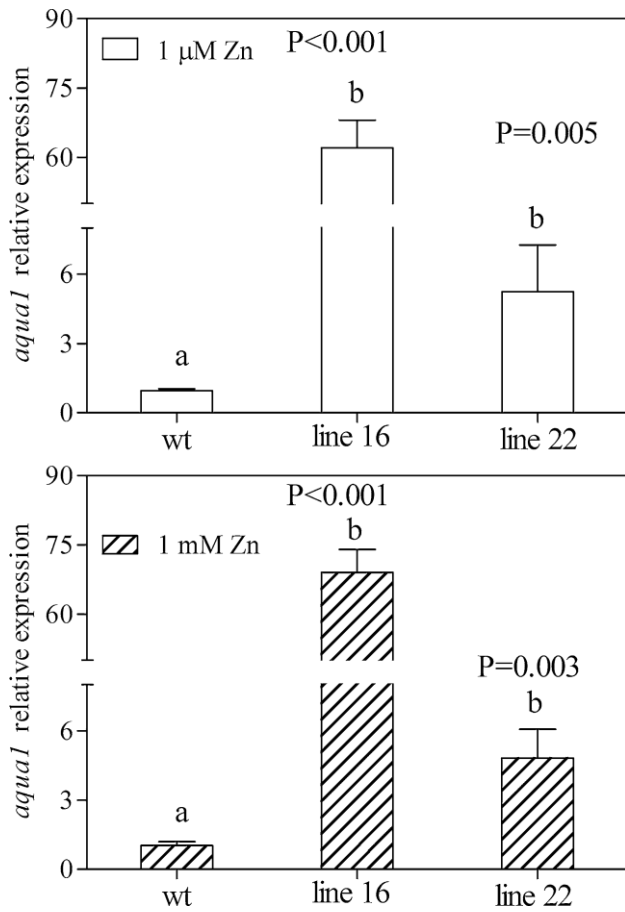


Fig. 2 Relative expression of *aqua1* transcripts in leaves of trans- genic plants (lines 16 and 22) compared to in vivo wt plants under 1 μM and 1 mM Zn treatments. Each histogram is the average of three independent biological samples with three technical replicates for each; error bars represent SD. *t* test analyses was performed on data of transgenic plants vs wt plants and letters denote differences among them

Concerning the fluorescence of Chl *a*, the maximum quantum yield of PSII (Fv/Fm) which gives the information about the proportion of the light absorbed by chlorophyll in PSII was reduced in apical leaves in all the lines exposed to Zn treatments (Fig. 7).

Comparison of growth parameters and Zn excess response among *P. alba* transgenic lines and wt plants

Transgenic lines and wt plants, grown under the same conditions in the control chamber, were phenotypically indistinguishable. Although *aqual* gene expression levels in line 16 and line 22 were different compared to wt, no morphological differences were observed (Fig. 3).

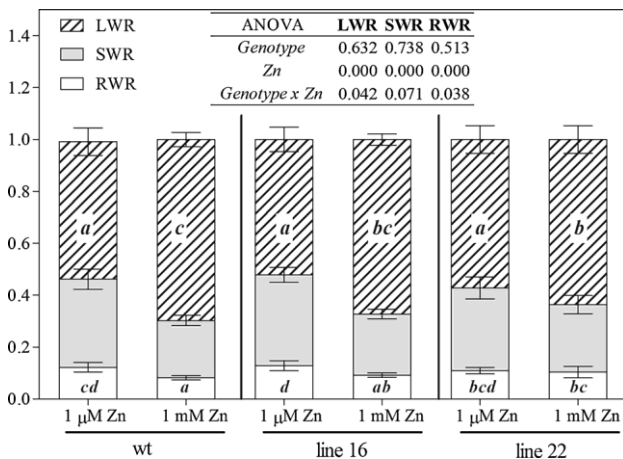


Fig. 3 Growth traits of *Populus alba* Villafranca clone in Control (1 μM Zn) and Zn treatments conditions (1 mM Zn), calculated after 35 days of growth on wt, over-expressing line 16 and line 22. Leaf Weight Ratio (LWR), Stem Weight Ratio (SWR), Root Weight Ratio (RWR) results were analyzed by two-way analysis of variance (ANOVA) and means ($n = 5$) were subjected to Tukey Multiple Comparison Test at 0.05 probability level. *Different letters* indicate difference among organs and treatments

Also, the leaf maturation process, estimated from the changes in chlorophyll content (SPAD measurements) in growing leaves, was not different among lines and Zn treatments (Fig. S1). Moreover, relative water content did not show differences among lines and Zn treatments (Fig. S2).

Over-expression of *aqual* in transgenic *Populus alba* increases growth rate as compared to wt plants under optimal growth conditions (Fig. 4a). The significant differences ($P < 0.05$) of RGR between the transgenic lines and the wt plants were detected in leaves and at the whole plant level, but for other organs (i.e., roots and stem) line 22 showed an intermediate phenotype, without significant differences from both the wt and the line 16.

In particular, in leaves of both transgenic lines there was an increase of RGR of *25 % as compared to wt plants, and at the whole plant level the increase is in the range of 21–26 %.

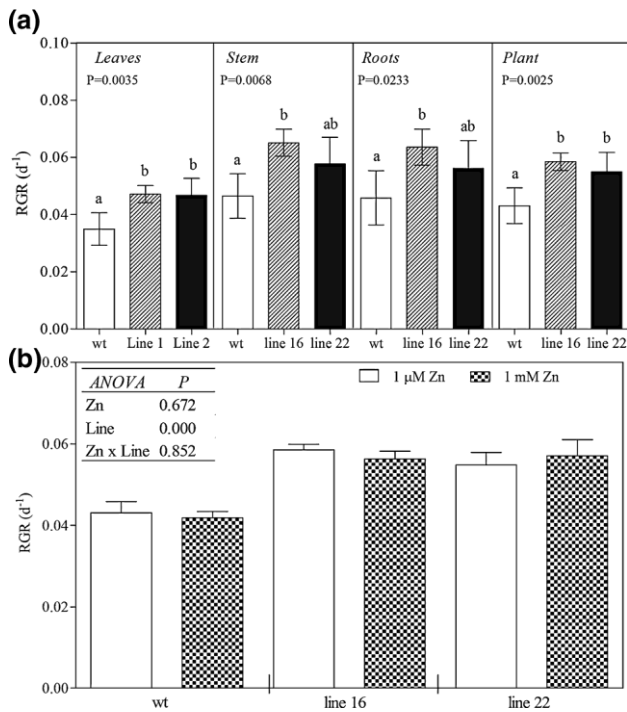


Fig. 4 Leaves, stem, roots, and wool plant relative growth rate (RGR) calculated after 35 days of 1 IM Zn treatment on wt, line 16 and line 22 *Populus alba* Villafranca clone; results were analyzed by one-way analysis of variance (ANOVA) and means were subjected to Tukey multiple comparison test at 0.05 probability level (a). Comparison of RGR calculated on *different lines* after 35 days of 1 IM and 1 mM Zn treatment; results were analyzed by two-way analysis of variance (ANOVA). Means ($n = 5$) were subjected to Tukey–Kramer multiple comparison test at 0.05 probability level (b)

For stem and roots, line 16 showed a significant increase of about 28 % in both plants parts as compared to wt plants, instead line 22 showed an increase (not significant) of *15 % for stem and *18 % for roots. 1 mM of Zn did not significantly affect plant RGR of either wt or transgenic plants (Fig. 4b). However, there was a significant effect ($P \setminus 0.001$) of the line factor with a mean increase in both transgenic lines of plant RGR (Fig. 4a, b), regardless of the treatment condition. The average increase of plant RGR in transgenic lines compared to wt, without considering the Zn treatments, was *26 % for line 16 and *24 % for line 22. The over-expression of *aqual* determines a slight increase of Zn concentration in leaves and roots in both transgenic lines. In particular, line 22 showed the higher Zn concentration (?17 % in leaves and ?15 % in roots in comparison to wt plants). However, these differences in response to excess Zn were not significant when compared to wt plants (Fig. 5a), suggesting that AQUA1 was probably not involved in Zn-transport. Significant differences in Tolerance Index (Ti) were found in roots of transgenic plants (Fig. 5b). Ti was significantly higher in line 22, with an increase of *40 % compared to wt plants. On the other hand, line 16 showed an intermediate phenotype with an increase *10 % that is not significantly different from both the wt and the other transgenic line.

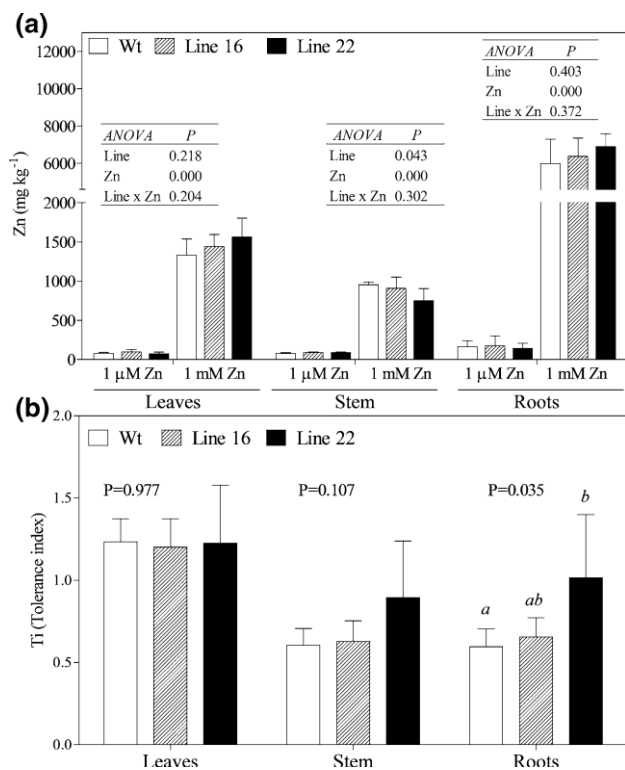


Fig. 5 Zn concentration (mg kg^{-1}) in leaves, stems, and roots of wt, over-expressing line 16 and line 22 of *Populus alba* Villafranca clone grown in hydroponic condition with 1 μM and 1 mM Zn for 35 days (a). Values are the means \pm standard deviation ($n = 5$). Results were analyzed by two-way analysis of variance (ANOVA). Means were subjected to Tukey–Kramer multiple comparison test at 0.05 probability level. Tolerance index calculated as ratio between the dry weight biomass of treatment (1 mM Zn) and control (1 μM Zn) conditions (b). The results were analyzed by one-way analysis of variance (ANOVA) and means were subjected to Tukey multiple comparison test at 0.05 probability level

Gas exchange and photosynthetic parameters

In order to understand the role of over-expression of *aqual* in transgenic *Populus alba* the photosynthetic parameters and chlorophyll *a* analyses were performed at the end of 35 days of treatments. A loss in photosynthetic capacity in treated plants was observed only in wt line (-67 %) when analyzed at ambient CO_2 (Table 1). The intrinsic Transpiration Efficiency calculated as ratio of net photosynthesis rate and g_s was significantly increased (2x) in transgenic *P. alba* line 16 and 22. Photosynthetic CO_2 response curves (A/C_i) did not change between treatments on wt and line 22 showing an increase when C_i concentration overlaps 600 ppm (Fig. 6).

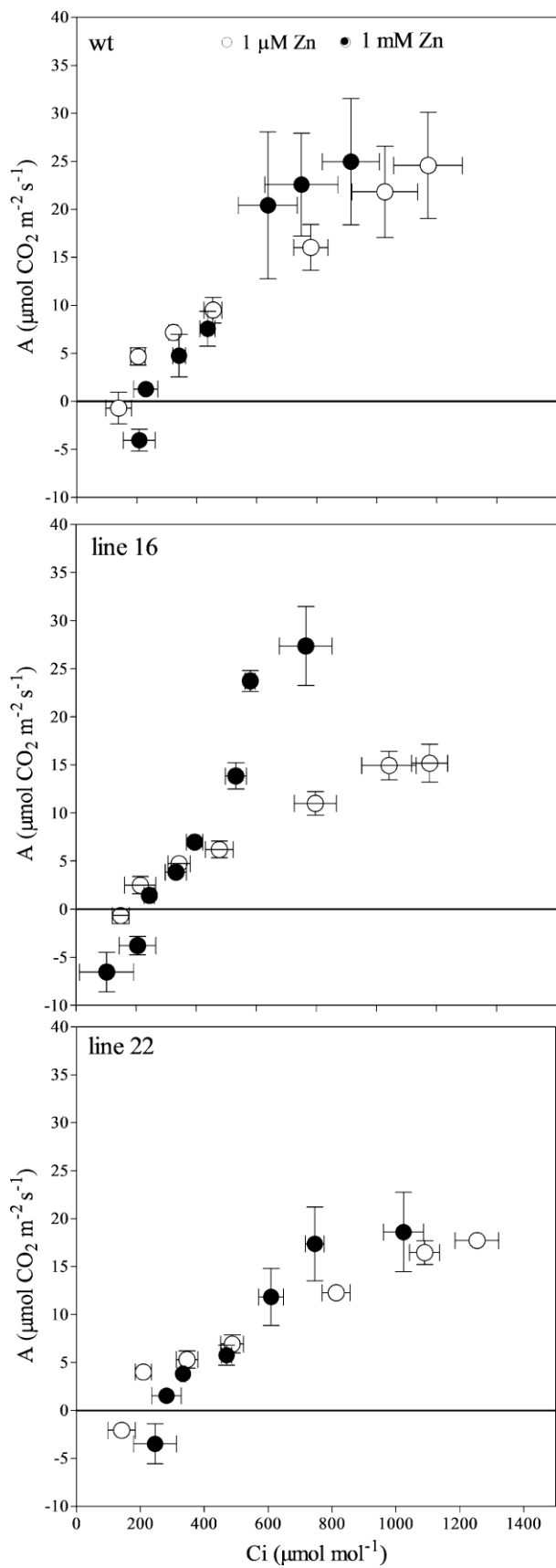


Fig. 6 Photosynthetic CO_2 response curves (A/C_i) determined for the first fully expanded leaf on plants grown in 1 μM and 1 mM Zn for 35 days. Each data are means of $n = 3$ analyses. Abbreviations: A CO_2 assimilation at saturating light level, C_i intercellular CO_2

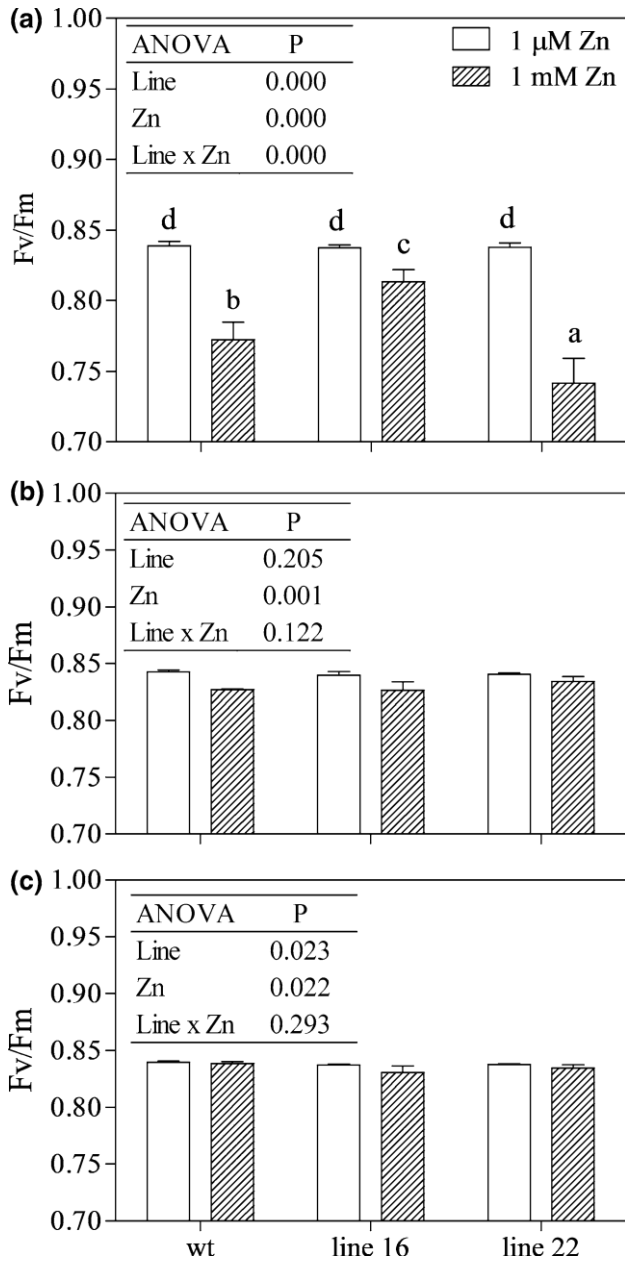


Fig. 7 Chlorophyll fluorescence parameter (Fv/Fm), analyzed on apical (a), median (b), and basal (c) leaves of *Populus alba* Villafranca clone grown in hydroponic condition with 1 μM and 1 mM Zn treatments for 35 days. Values are means ± standard deviation (n = 5). Results were analyzed by two-way analysis of variance (ANOVA). Means were subjected to Fisher's LSD test at 0.05 probability level. Different letters represent significant differences among treatments and line interaction

Element distributions in plants in relation to Zn uptake

The PCA results indicate that mineral nutrients have a distinct response pattern in leaves, stem, and root tissues but the response pattern is similar in wt and transgenic lines. PCA conducted on nutrients data produced two significant PCs that, together, explain 86.5 % of the total variance in the data and are retained for further analysis. The other nine components will not be discussed further because their eigenvalues are less than one and they consequently explain little of the variability in the dataset. B, Ca, and N show a similar distribution pattern to S, Mg, and K (Fig. 8). Each element has a high loading value on the first principal component (which explains 52.28 % of the total variance). P, Fe, Mn, and Cu are positively associated with the second component (34.28 % of the variance) (Fig. 8 and Table S1). Once the association between the 10 elements and the two components is identified, the scores assigned to individual lines with respect to each component are analyzed in order to identify common patterns. A scatterplot of the scores of different line samples in a Fact.1/Fact.2 score space and are presented in Fig. 9. The scores are marked by their line names and related organs to identify possible differences in elements distribution between lines and organs. In the PCA scatterplot is possible to see a different organ and treatments distribution of the data, while no differences were detected between lines.

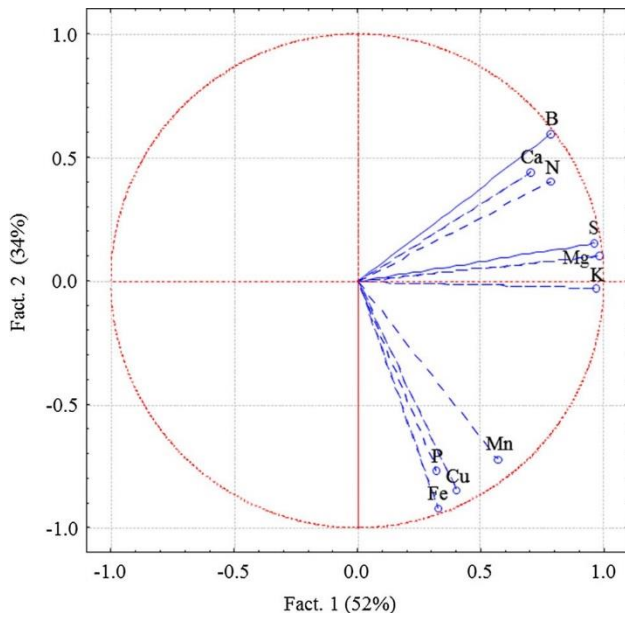


Fig. 8 PCA scores for different elements on lines (wt, line 22 and line 16) and organs (leaves, stem, and roots)

Fig. 9 PCA scores plot of nutrients data of *Populus alba* Villafranca clone grown in hydroponic condition with 1 μ M and 1 mM Zn treatments, indicating clustering of WT (wild type), line 16 (GM16) and line 22 (GM22) leaf, roots, and stem samples. The square represent treated clone, the circle identify control one. Different lines are reported in association with organs (R roots, S stem, L leaves)

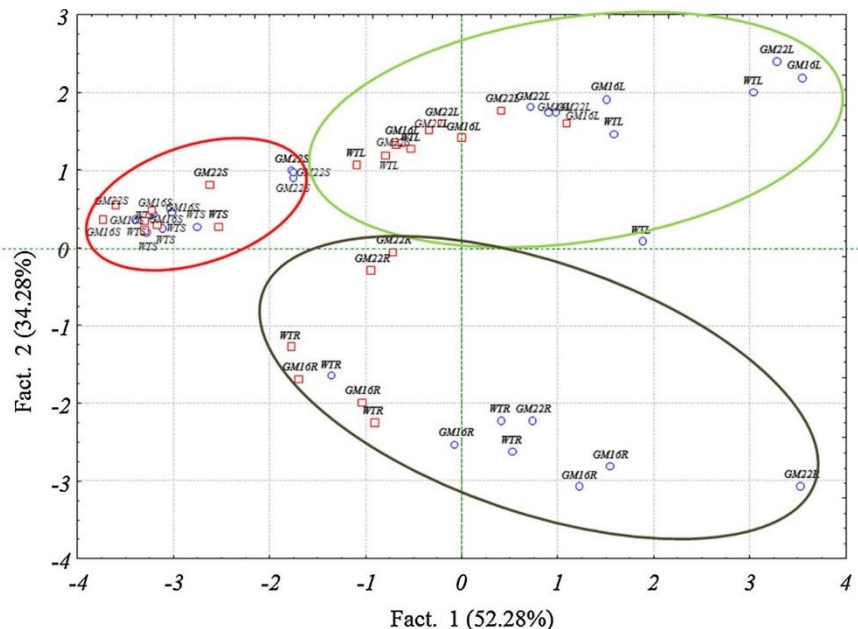


Table 1 Foliar gas exchange parameters estimated to ambient CO₂ (395 ± 20 ppm) in different lines of *Populus alba* Villafranca clone grown in hydroponic condition with 1 lM and 1 mM Zn treatments

	Line			ANOVA		
	wt	16	22	Line	Zn	Line 9 Zn
iTE						
1 lM Zn	0.050±0.0023a	0.040±0.0099a	0.035±0.0077a	0.398	0.017	0.059
1 mM Zn	0.041±0.0112a	0.080±0.0296b	0.071 ± 0.0224b			
g _s						
1 lM Zn	143 ± 14.8	120 ± 3.6	151 ± 24.8	0.757	0.000	0.157
1 mM Zn	63 ± 21.1	72 ± 31.9	57 ± 13.8			
A						
1 lM Zn	7.2 ± 0.95c	4.7 ± 1.06a	5.3 ± 1.56a	0.811	0.001	0.003
1 mM Zn	2.4 ± 0.32a	5.2 ± 1.53a	3.8 ± 0.39a			

Values are mean ± standard deviation ($n = 5$). Results were analyzed by two-way analysis of variance (ANOVA). Means were subjected to Fisher's LSD test at 0.05 probability level. Different letters represent significant differences among treatments and line interaction. Intrinsic Transpiration Efficiency (iTE) calculated as ratio of A and g_s

A CO₂ assimilation at saturating light level (1mol CO₂ m⁻² s⁻¹), g_s stomatal conductance to water vapor (mmol H₂O m⁻² s⁻¹)

Discussion

Plant aquaporins are involved in almost every aspect of plant life cycle such as cell growth and elongation, transpiration, seed germination, and response to biotic and abiotic stresses (Li et al. 2013). Thanks to expression studies and the use of transgenic plants, the physiological roles of several aquaporins have been elucidated in the last few years. For instance, the expression of *Arabidopsis AtTIP1;1* is induced by gibberellic acid (GA3), a phyto-hormone regulating cell growth, and its expression is strongly correlated with an increased cell size in different tissues (Ludevid et al. 1992; Philips and Huttly 1994). Moreover, over-expression in transgenic plants of *Arabidopsis AtPIP1;2* and *Panax ginseng PgTIP1* significantly increased plant growth rate when compared to wt plants (Aharon et al. 2003; Peng et al. 2007). Since the over-expression of *aqual* in transgenic Villafranca clones induced a general increase of plants growth rate (i.e., RGR), in comparison to wt plants, we suggest a major role of this aquaporin in regulation of plant growth and conferring better intrinsic transpiration efficiency.

The increased growth rate of transgenic plants over-expressing aquaporin genes has been related to increased mesophyll and stomatal conductance, resulting then in an increased CO₂ assimilation and carbon fixation (Aharon et al. 2003). CO₂ is assimilated through stomata that rapidly regulates their opening and closing by water movement between guard cells and the surrounding tissues (Roelfsema and Hedrich 2005). Specific guard cells localized aquaporins expression have been previously detected for several species such as *Arabidopsis* and *N. tabacum* (reviewed in Heinein et al. 2009). In sunflower, the expression of *SunTIP7* transcripts were increased during stomatal closure (Sarda et al. 1997), suggesting the involvement of aquaporin in regulating guard cell water flux, and stomatal opening. In addition, some aquaporins showed also the ability to facilitate CO₂ transport when expressed in *Xenopus* oocytes and in yeast cells (Heckwolf et al. 2011; Uehlein et al. 2003). Several studies, using over-expressing/silencing aquaporin mutants, showed also a positive correlation between the expression of these aquaporins and mesophyll conductance in different plant species, like rice, tobacco, and *Arabidopsis* (Heckwolf et al. 2011; Flexas et al. 2006; Hanba et al. 2004). The localization of *aqual* in guard cell in transgenic *P. alba* plants could suggest a possible involvement of this

aquaporin in regulation of stomatal gating. However, the over-expression of *aqual* did not significantly affect stomatal conductance in controlled conditions at simulated atmospheric CO₂ and also did not affect fluorescence parameters. This could be explained by a possible buffering of *aqual* over-expression by post-translational regulation of its activity, and/or sub-cellular localization, in order to avoid an excessive transpiration. Indeed, in *V. faba* guard cell volume is regulated by kinase-dependent mechanisms involved in membrane trafficking (Shope and Mott 2006), such as phosphatidylinositol-3-kinase activity, that regulate also the sub-cellular localization of *aqual* in *Arabidopsis* protoplasts (Andreucci et al. 2015). Thus, it could be plausible that similar mechanisms could occur also in *P. alba aqual* over-expressing lines, resulting then in a similar stomatal conductance between transgenic and wt plants. On the other hand, it could be also possible that *aqual* could be involved mainly in cell growth and cell elongation, instead of regulating transpiration as also demonstrated by an increased RGR in transgenic lines.

In the past years, several aquaporins have been associated with response to different environmental stresses (Li et al. 2013; Maurel et al. 2008), probably through regulation of plant water status and transpiration. However, different studies showed contrasting results of the role of aquaporin in response to stress, depending mainly on the plant species and the stress under investigation. Indeed, the over-expression of aquaporins in transgenic plants could confer both higher tolerance, or higher sensitivity to stresses, probably by modifying plant-water relations (Li et al. 2013). Also heavy metal toxicity could affect water absorption and transport at the root (Prasad 1997; Barceló et al. 1986), but can also reduce leaf transpiration and water potential (Pandey and Sharma 2002). In addition, some aquaporins have been shown to directly transport heavy metals or to confer resistance to this stress when over-expressed in plants (Hove and Bhave 2011; Negishi et al. 2012; Zhang et al. 2008). Since *aqual* is down-regulated in response to Zn excess in *P. x euramericana* I-214 leaves and roots (Di Baccio et al. 2011), we investigated the effect of its over-expression in response to excess Zn in *P. alba* over-expressing lines.

When exposed to Zn excess, the transgenic lines did not show any significant increase in Zn accumulation in roots and leaves, even though there was a slight increase in comparison to wt plants. Mineral elements analyzed by PCA, on the contrary, showed that Zn induces a reduction on other elements uptake but also in this case not related to factor line. In particular, the different responses of leaves and root analyzed reveal a distinct associations between PC1 and the mineral nutrients scores developed from PCA analysis. These results could be explained by the different physiological role of these organs. Since the over-expression of genes involved in heavy metal transport/homeostasis usually drastically increase metal accumulation in transgenic over-expressing plants (Sinclair and Krämer 2012; Adams et al. 2011), these results could suggest that *aqual* is not directly involved in Zn transport and homeostasis, but mainly in plant

growth. The down-regulation of *aqual* in response to excess Zn, showed by Di Baccio et al. (2011), is probably a mechanism for reducing plant growth in response to Zn stress, in order to avoid excessive accumulation of this metal.

Our results suggest that a general increase in water transport was detected in *Populus alba* transgenic plants. The Villafranca transgenic plants showed an increased tolerance, expressed as biomass production in excess Zn and control condition, toward excess Zn in roots. Anyway, the Villafranca clone did not show significant differences in Zn accumulation and partitioning between wt and transgenic plants. These results could suggest that *aqual* did not transport Zn and that the tolerance in roots tissues could be related to the increased growth rate shown by transgenic plants compared to wt.

Author contribution statement Andrea Ariani has contributed to the planning of the experiment, data collection and elaboration (transformation, gene expression), manuscript draft, and its final approval. Alessandra Francini has contributed to the planning of the experiment, data collection and elaboration (Zn concentration, chlorophyll fluorescence analyses, gas exchange measurement), manuscript draft, and its final approval. Andrea Andreucci has contributed to the planning of the experiment, data collection and elaboration (gene expression, confocal imaging collection), manuscript draft, and its final approval. Luca Sebastiani has contributed to the planning of the experiment, data collection and elaboration (Zn concentration, gene expression, statistical elaboration) of data manuscript draft, and its final approval.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Adams JP, Adeli A, Hsu CY, Harkess RL, Page GP, dePamphilis CW, Schultz EB, Yuceer C (2011) Poplar maintains zinc homeostasis with a heavy metal genes HMA4 and PCS1. *J Exp Bot* 62:3737–3752
- Agree P, Saboori AM, Asimos A, Smith BL (1987) Purification and partial characterization of the Mt 30,000 integral membrane protein associated with the erythrocyte Rh(D) antigen. *J Biol Chem* 262:17497–17503
- Aharon R, Shahak S, Bendov R, Kapulnik Y, Galili G (2003) Overexpression of a plasma membrane aquaporin in transgenic tobacco improves plant vigour under favourable growth conditions but not under drought or salt stress. *Plant Cell* 15:439–447
- Andreucci A, Ariani A, Sebastiani L (2015) *In vitro* functional characterization of AQUA1: a new poplar (*Populus x euramericana* clone I-214) aquaporin involved in Zinc stress. International Plant Science Conferences (IPSC) from nature to Technological Exploitations. Florence 2–5 September 2014. Abstract p xviii
- Ariani A, Gepts P (2015) Genome-wide identification and characterization of aquaporin gene family in common bean (*Phaseolus vulgaris* L.). *Mol Genet Genomics*. doi:10.1007/s00438-015-1038-2
- Arnon DI, Hoagland DR (1940) Crop production in artificial culture solutions and in soils with special reference to factors influencing yields and absorption of inorganic nutrients. *Soil Sci* 50:463–485
- Bae EK, Lee H, Lee JS, Noh EW (2011) Drought, salt and wounding stress induce the expression of the plasma membrane intrinsic protein 1 gene in poplar (*Populus alba* 9 *P. tremula* var. *glandulosa*). *Gene* 483:43–48
- Barceló J, Poschenrieder C, Andreu I, Gunse B (1986) Cadmium-induced decrease of water stress resistance in bush bean plants (*Phaseolus vulgaris* L. cv. Contender) I. Effects of Cd on water potential, relative water content and cell wall elasticity. *J Plant Physiol* 125:17–25
- Besse M, Knipfer T, Miller AJ, Verdeil J-L, Jahn TP et al (2011) Developmental pattern of aquaporin expression in barley (*Hordeum vulgare* L.) leaves. *J Exp Bot* 62:4127–4142
- Brunner AM, Yakovlev IA, Strauss SH (2004) Validating internal controls for quantitative plant gene expression studies. *BMC Plant Biol* 4:14. doi:10.1186/1471-2229-4-14
- Chaumont F, Barrieu F, Wojcik E, Chrispeels MJ, Jung R (2001) Aquaporins constitute a large and highly divergent protein family in maize. *Plant Physiol* 125:1206–1215
- Cohen D, Bogeat-Triboulot MB, Vialet-Chabrand S, Merret R, Courty PE, Moretti S, Bizet F, Guillot A, Hummel I (2013) Developmental and environmental regulation of aquaporin gene expression across *Populus* species: divergence or redundancy? *PLoS One* 8:e55506
- Confalonieri M, Belenghi B, Balestrazzi A, Negri S, Faccioto G, Schenone G, Delledonne M (2000) Transformation of elite white poplar (*Populus alba* L.) cv. ‘Villafranca’ and evaluation of herbicide resistance. *Plant Cell Rep* 19:978–982
- Cui XH, Hao FS, Chen H, Chen J, Wang XC (2008) Expression of the *Vicia faba* VPIPI1 gene in *Arabidopsis thaliana* plants improves their drought resistance. *J Plant Res* 121:207–214
- Di Baccio D, Tognetti R, Minnoci A, Sebastiani L (2009) Responses of the *Populus* 9 euramericana clone I-214 to excess zinc: carbon assimilation, structural modifications, metal distribution and cellular localization. *Environ Exp Bot* 67:153–163
- Di Baccio D, Galla G, Bracci T, Andreucci A, Barcaccia G, Tognetti R, Sebastiani L (2011) Transcriptome analyses of *Populus* 9 euramericana clone I-214 leaves exposed to excess zinc. *Tree Physiol* 31:1293–1308
- Dickmann DI (1972) Photosynthesis and respiration by developing leaves of cottonwood (*Populus deltoids* Bartr.). *Bot Gaz* 132:253–259
- Du J, Miura E, Robischon M, Martinez C, Groover A (2011) The *Populus* class II HD ZIP transcription factor POPCORONA affects cell differentiation during secondary growth on woody stems. *PLoS One* 6:e17458
- Flexas J, Ribas-Carbó M, Hanson DT, Bota J, Otto B, Cifre J, McDowell N, Medrano H, Kaldenh R (2006) Tobacco aquaporin NtAQP1 is involved in mesophyll conductance to CO₂ in vivo. *Plant J* 48:427–439

- Francini A, Nali C, Picchi V, Lorenzini G (2007) Metabolic changes in white clover clones exposed to ozone. *Env Exp Bot* 60:11–19
- Gupt AB, Sankararamkrishnan R (2009) Genome wide analysis of major intrinsic proteins in the tree plant *Populus trichocarpa*: characterization of XIP subfamily of aquaporins from evolutionary perspective. *BMC Plant Biol* 9:134
- Hacke UG, Plavcova L, Almeida-Rodriguez A, King-Jones S, Zhou WC et al (2010) Influence of nitrogen fertilization on xylem traits and aquaporin expression in stems of hybrid poplar. *Tree Physiol* 30:1016–1025
- Hanba YT, Shibasaka M, Hayashi Y, Hayakawa T, Kasamo K, Terashima I, Katsuhara M (2004) Overexpression of the barley aquaporin HvPIP2;1 increase internal CO₂ conductance and CO₂ assimilation in the leaves of transgenic rice plants. *Plant Cell Physiol* 45:521–529
- Heckwolf M, Pate DT, Keldenhoff R (2011) The *Arabidopsis thaliana* aquaporin AtPIP1;2 is a physiological relevant CO₂ transport facilitator. *Plant J* 67:795–804
- Heinein RB, Ye Q, Chaumont F (2009) Role of aquaporins in leaf physiology. *J Exp Bot* 60:2971–2985
- Hove RM, Bhavne M (2011) Plant aquaporins with non-aqua functions: deciphering the signature sequences. *Plant Mol Biol* 75:413–430
- Hunt R (1978) Plant growth analysis. Camelot Press Ltd, Southampton Kjeldahl J (1883) Neue methode zur bestimmung des stickstoffs in organischen körpern (New method for the determination of nitrogen in organic substances). *Z Anal Chem* 22(1):366–383
- Kohler A, Delaruelle C, Martin D, Encelot N, Martin F (2003) The poplar root transcriptome: analysis of 7000 expressed sequence tags. *FEBS Lett* 542:37–41
- Lee SH, Chung GC, Jang JY, Ahn SJ, Zwiazek JJ (2012) Overexpression of PIP2;5 aquaporin alleviates effects of low root temperature on cell hydraulic conductivity and growth in *Arabidopsis*. *Plant Physiol* 159:479–488
- Li GW, Zhang MH, Cai WM, Sun WN, Su WA (2008) Characterization of OsPIP2;7, a water channel protein in rice. *Plant Cell Physiol* 49(12):1851–1858. doi:10.1093/pcp/pcn166
- Li G, Santoni V, Maurel C (2013) Plant aquaporins: roles in plant physiology. *Biochim Biophys Acta*. doi:10.1016/j.bbagen.2013. 11.004
- Lloyd G, McCown B (1981) Commercially-feasible micropropagation of Mountain laurel, *Kolmia latifolia*, by use of shoot tip culture. *Inter Plant Propag Soc* 30:421–427
- Lopez D, Bronner G, Brunel N, Auguin D, Bourgerie S et al (2012) Insights into *Populus* XIP aquaporins: evolutionary expansion, protein functionality, and environmental regulation. *J Exp Bot* 63:2217–2230
- Ludevid D, Höfte H, Himelblau E, Chrispeels MJ (1992) The expression pattern of the tonoplast intrinsic protein c-TIP in *Arabidopsis thaliana* is correlated with cell enlargement. *Plant Physiol* 100:1633–1639
- Marjanovic Z, Uehlein N, Kaldenhoff R, Zwiazek JJ, Weiss M et al (2005) Aquaporins in poplar: what a difference a symbiont makes! *Planta* 222:258–268
- Matsumoto T, Hian HL, Su WA, Tanaka D, Liu C, Iwasaki I, Kitagawa Y (2009) Role of the aquaporin PIP1 subfamily in the chilling tolerance of rice. *Plant Cell Physiol* 50:216–229
- Maurel C (1997) Aquaporins and water permeability of plant membranes. *Annu Rev Plant Physiol Plant Mol Biol* 48:399–429
- Maurel C, Verdoucq L, Luu DT, Santoni V (2008) Plant aquaporins: membrane channels with multiple integrated functions. *Ann Rev Plant Biol* 59:595–624
- Nakagawa T, Nakamura S, Tanaka K, Kawamukai M, Suzuki T, Nakamura K, Kimura T, Ishiguro S (2008) Development of R4 gateway binary vectors (R4pGWB) enabling high-throughput promoter swapping for plant research. *Biosci Biotechnol Biochem* 72:624–629
- Negishi T, Oshima K, Hattori M, Kanai M, Mano S, Nishimura M, Yoshida K (2012) Tonoplast- and plasma membrane-localized aquaporin-family transporters in blue hydrangea sepals of aluminum hyperaccumulating plant. *PLoS One* 7:e43189
- Pandey N, Sharma CP (2002) Effect of heavy metals Co²⁺, Ni²⁺ and Cd²⁺ on growth and metabolism of cabbage. *Plant Sci* 163:753–758
- Peng Y, Lin W, Cai W, Arora R (2007) Over-expression of a *Panax ginseng* tonoplast aquaporin alters salt tolerance, drought tolerance and cold acclimation ability in transgenic *Arabidopsis* plants. *Planta* 226:729–740
- Perrone I, Gambino G, Chitarra W, Vitali M, Pagliarini C et al (2012) The grapevine root-specific aquaporin VvPIP2;4N control root hydraulic conductance and leaf gas exchange under well-watered conditions but not under water stress. *Plant Physiol* 160:956–977
- Philips AL, Huttly AK (1994) Cloning of two gibberellin-regulated cDNAs from *Arabidopsis thaliana* by subtractive hybridization: expression of the tonoplast water channel, c-TIP, is increased by GA3. *Plant Mol Biol* 24:603–615
- Postaire O, Tornaire-Roux C, Grondin A, Boursiac Y, Morillon R, Schäffner AR, Maurel C (2010) A PIP1 aquaporin contributes to hydrostatic pressure-induced water transport in both the root and rosette of *Arabidopsis*. *Plant Physiol* 152:1418–1430
- Prasad MNV (1997) Trace metals. In: Prasad MNV (ed) *Plant ecophysiology*. New York, USA, pp 207–249
- Quigley F, Rosenberg JM, Shachar-Hill Y, Bohnert HJ (2001) From genome to function: the *Arabidopsis* aquaporins. *Genome Biol* 3:1–17
- Robinschon M, Du J, Miura E, Groover A (2011) The *Populus* class II HD ZIP, popREVOLUTA, influence cambium initiation and patterning of woody stems. *Plant Physiol* 155:1214–1225
- Rodgers-Melnick E, Mane SP, Dharmawardhana P, Slavov GT, Crasta OR et al (2012) Contrasting patterns of evolution following whole genome versus tandem duplication events in *Populus*. *Genom Res* 22:95–105
- Roelfsema MRG, Hedrich R (2005) In the light of stomatal opening: new insight into ‘the Watergate’. *New Phytol* 167:665–691
- Romeo S, Francini A, Ariani A, Sebastiani L (2014a) Phytoremediation of Zn: identify the diverging resistance, uptake and biomass production behaviours of poplar clones under high zinc stress. *Water Air Soil Pollut*. doi:10.1007/s11270-013-1813-9
- Romeo S, Trupiano D, Ariani A, Renzone G, Scippa GS, Scaloni A, Sebastiani L (2014b) Proteomic analysis of *Populus 9 euramericana* (clone I-214) roots to identify key factors involved in zinc stress response. *J Plant Physiol* 171:1054–1063
- Romeo S, Lombardi L, Andreucci A, Lux A, Stephen Horner D, Sebastiani L (2015) RNA sequencing of *Populus x canadensis* roots identifies key molecular mechanisms underlying physiological adaptation to excess zinc. *PLoS One*. doi:10.1371/journal.pone.0117571
- Sade N, Vinocur BJ, Diber A, Shatil A, Ronen G, Rissan H, Wallach R, Karchi H, Moshelion M (2009) Improving plant stress tolerance and yield production: is the tonoplast aquaporin SITIP2;2 a key to isohydric to anisohydric conversion? *New Phytol* 181:651–661
- Sakurai J, Ishikawa F, Yamaguchi T, Uemure M, Maeshima M (2005) Identification of 33 rice aquaporin genes and analysis of their expression and function. *Plant Cell Physiol* 26:1568–1577
- Sarda X, Tousch D, Ferrare K, Legrand E, Dupuis JM, Casse-Delbart F, Lamaze T (1997) Two TIP-like genes encoding aquaporins are expressed in sunflower guard cells. *The Plant J* 12:1103–1111
- Secchi F, Zwieniecki MA (2010) Patterns of PIP gene expression in *Populus trichocarpa* during recovery from xylem embolism suggest a major role for the PIP1 aquaporin subfamily as moderators of refilling process. *Plant Cell Environ* 33:1285–1297
- Shope JC, Mott KA (2006) Membrane trafficking and osmotically induced volume changes in guard cells. *J Exp Bot* 57:4123–4413

- Sinclair SA, Krämer U (2012) The zinc homeostasis network of land plant Roes. *Biochim Biophys Acta* 1823:1553–1567
- Song J, Lu S, Chen ZZ, Lourenco R, Chiang VL (2006) Genetic transformation of *Populus trichocarpa* genotype Nisqually-1: a functional genomic tool for woody plants. *Plant Cell Physiol* 47:1582–1589
- Su X, Chu Y, Li H, Hou Y, Zhang B et al (2011) Expression of multiple resistance genes enhances tolerance to environmental stressors in transgenic poplar (*Populus x euramericana* 'Guariento'). *PLoS One* 6:e24614
- Tuskan GA, Difazio S, Jansson S, Bohlmann J, Grigoriev I et al (2006) The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science* 313:1596–1604
- Uehlein N, Lovisolo C, Siefritz F, Kaldenhoff R (2003) The tobacco aquaporin ntAQP1 is a membrane CO₂ pore with physiological functions. *Nature* 425:734–737
- Wilkins DA (1957) A technique for the measurement of lead tolerance in plants. *Nature* 180:37–38
- Wullschlegel SD, Weston DJ, DiFazio SP, Tuskan GA (2012) Revisiting the sequencing of the first tree genome: *populus trichocarpa*. *Tree Physiol* 33:357–364
- Zhang Y, Ma K, Sadana P, Chowdhury F, Gaillard S, Wang F, McDonnell DP, Unterman TG, Elam MB, Park EA (2006) Estrogen related receptors stimulate pyruvate dehydrogenase kinase isoform 4 (PDK4) gene expression. *J Biol Chem* 281(52):39897–39906
- Zhang Y, Wang Z, Chai T, Wen Z, Zhang H (2008) Indian mustard aquaporin improves drought and heavy-metal resistance in tobacco. *Mol Biotechnol* 40:280–292