

Water Restriction Alters the Expression of Calcium Transporters in Eucalypt Roots

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Research Article

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Abstract

Background and Aims

Calcium (Ca) plays an important role to increase drought tolerance in plants. We evaluated the effect of water restriction and Ca supply on the expression of Ca transporters, on Ca acquisition, allocation and its effects in eucalypt responses to drought.

Methods

Drought-tolerant eucalyptus plants (*Eucalyptus urophylla* x *Eucalyptus camaldulensis* - clone VM01) were grown in a hydroponic system in the presence or absence of Ca and subjected or not to water restriction using polyethylene glycol. Ca concentration by plants, gas exchange, plant growth, and the expression of the *ACA11* and *MCA1* Ca transporters were evaluated.

Results

Water restriction increased the expression of *ACA11* (vacuole Ca transporter) and decreased the expression of *MCA1* (endodermal Ca transporter), in eucalypt roots. Water restriction increased Ca content in plant roots and Ca supply ameliorated the negative effects of water stress.

Conclusion

Water restriction alters the expression of Ca transporters in eucalypt roots altering the Ca allocation pattern and improving root growth and water status. Although caution must be taken, our findings bring novel evidence of an important mechanism that favors survival of drought-tolerant woody plants under water deficit.

Introduction

Calcium (Ca) is essential for plant growth and development (Sharma et al. 2017). Along with several key functions in plants, this macronutrient is an important component of pectins (Demarty et al. 1984), regulates stomatal movement, participates in multiple photosynthetic pathways (Wang et al. 2019), reduces oxidative damage and has a role in osmoregulation and hydraulic conductivity (Wang et al. 2014; Li et al. 2015; Naeem et al. 2017; Kang et al. 2017). Furthermore, Ca is a secondary messenger in plant responses to distinct biotic and abiotic stresses (Thor 2019; Iqbal et al. 2021).

Understanding the mechanisms controlling Ca acquisition by plants is essential to better comprehend plant responses to environmental changes. A relationship between Ca content and increased drought tolerance has been observed in different plant species (Ahanger et al. 2016; Naeem et al. 2017; Kang et al.

2017; Wang and Komatsu 2017; Müller et al. 2017; Leng et al. 2020). However, the molecular mechanisms driving an increased Ca uptake and accumulation in plants under drought still remain unclear.

The uptake of Ca by root cells is mediated by permeable transporters located in the plasma membrane (White 2000; White and Broadley 2003b). Once inside the roots, Ca^{2+} ions can be translocated to shoots or stored in the cell wall and, or organelles (Demarty et al. 1984; Pinto and Ferreira 2015; Kuronuma and Watanabe 2021). The vacuole is considered the largest Ca storage deposit in plant cells (Peiter 2011; Pinto and Ferreira 2015) and the class of vacuolar transporters that controls Ca influx into this organelle is called self-inhibited Ca-ATPases (ACAs) (Schönknecht 2013; García Bossi et al. 2020). ACA11 is located in the tonoplast of large vacuoles of root epidermal cells, favoring the influx of Ca from the cytoplasm (Pittman et al. 2011; Pinto and Ferreira 2015; García Bossi et al. 2020). However, to the best of our knowledge, there are no studies demonstrating changes in the expression of *ACA11* under water limitation.

Ca can be stored in root cells or translocated to shoots through apoplastic and symplastic pathways (White and Broadley 2003b; Thor 2019). The Ca displacement through these two pathways is well balanced. A common model suggests that it moves through the apoplastic pathway via cortex cells until it reaches the endoderm (White 2001), where the influx into the cytosol of endodermal cells is an essential step for the Ca to reach the xylem (White 2000; White and Broadley 2003a; Kumar et al. 2015). In *Arabidopsis thaliana*, the mechanosensitive channel protein permeable to Ca^{2+} (MCA1), located in the plasma membrane of endodermal root cells, is suggested as a strong candidate to participate in this process (Nakagawa et al. 2007; Yamanaka et al. 2010).

Drought episodes have been considered one of the main causes of tree mortality in several regions of the world (Choat et al. 2018; Gazol and Camarero 2022; Bauman et al. 2022). However, mechanisms driving an increased Ca uptake and accumulation in trees during drought are still unclear. In this study, we addressed the effect of water restriction on Ca acquisition and accumulation in a drought-tolerant eucalypt genotype and unveiled at least part of the mechanisms that regulates this process.

Material And Methods

2.1 Plant growth conditions and experimental design

Three-month-old seedlings (~ 30 cm long) of the VM01 eucalyptus clone (*Eucalyptus urophylla* x *Eucalyptus camaldulensis*) were used. This clone was previously characterized as highly tolerant to drought (Martins et al. 2018; Santos et al. 2020) and has been shown to accumulate high amounts of Ca in roots (data not published). The seedlings were acclimatized in a greenhouse, in polyethylene pots (3 L) containing Clark's nutrient solution (Clark 1982), modified by Locatelli et al. (1984), under constant aeration and pH near 6. In the initial phase of the acclimatization period, the culture solution had an ionic

strength equivalent to 50% of the final solution. The ionic strength of the solution was gradually raised to 100% of the standard solution. Solutions were changed on a weekly basis (Pinto et al. 2011).

After 15 days of acclimatization, the plants were submitted to treatments with presence or absence of Ca for 12 days. After this period, water restriction was imposed by using polyethylene glycol (PEG 6000) in the plant culture solution. The 235g dose of PEG 6000 was gradually added in an interval of ten days until the water potential of the solution reached -0.65 MPa (Michel and Kaufmann, 1973). Plant material was collected six days after the solution reached the -0.65 MPa water potential, that is, 16 days after the start of the water restriction imposition. The nutrient replacement solution was changed every six days. The experiment was designed in randomized blocks, in a factorial arrangement, (2×2) : water comfort (WC) or water restriction (WR) $\times$ presence of Ca (+Ca) or absence of calcium (-Ca), with five replicates.

2.2 Water status and photosynthesis

Predawn leaf water potential (Ψ_w) was determined in the early morning (between 4 and 5 A.M.) in fully expanded leaves, using a "Scholander" type pressure chamber (PMS-1000; PMS Instruments, Albany, NY, USA). The net photosynthetic assimilation rate (A), stomatal conductance (g_s), transpiration (E) and the ratio between the internal and external concentration of CO_2 (C_i/C_a) were determined using an infra-red gas analyzer (IRGA L Crop-SD). Measurements were performed in the morning on fully expanded leaves of the third node between 08:00 am and 12:30 pm, using constant photosynthetically active radiation ($1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and atmospheric CO_2 concentration.

2.3 Determination of Ca concentration, accumulation and translocation efficiency

Samples of shoots and roots were collected at the end of the experiment (16th day) and dried in an oven with forced air circulation at a temperature of 65°C until reaching constant weight. Subsequently, the shoots and roots were ground in a Wiley mill, passed through a 0.5 mm mesh sieve and subjected to nitric-perchloric digestion (Johnson and Ulrich 1959). The Ca concentration was determined by inductively coupled plasma optical emission (ICP-OES Horiba, Ultima Expert) and expressed as mg g^{-1} dry mass. The Ca accumulation was calculated by multiplying the Ca concentration by the dry mass of the root or shoot and expressed as mg plant^{-1} (root or shoot). The calcium translocation efficiency (TE) was calculated as the Ca accumulation in shoots divided by Ca accumulation in the plant (Li et al. 1991).

2.4 Morphological features

The morphological characteristics of the root system (length, volume, surface area and average diameter of the roots) were obtained using the image analysis system WinRHIZO® Software (WinRHIZO Pro, Regent Inc. Instr., Canada). The total leaf area was determined using a leaf area meter (LI-3100C; LI-COR BIOSCIENCES INC., 2004). Shoot and root samples were placed into paper bags and oven-dried (65°C) until reaching constant mass and weighed to determine the shoot dry mass (SDM), root dry mass (RDM) and these root and shoot dry matter ratio (RDM/SDM).

2.5 Analysis of the gene expression of Ca transporters

Gene expression analyses were performed on samples of root apices with approximately 5 cm in length. The material was collected and immediately placed in liquid nitrogen, stored at -80°C and subsequently macerated using a mortar and pestle. Total RNA was extracted from the roots of four biological replicates according to Chang et al. (1993), with a few modifications. The prepared extraction buffer contained 2% CTAB, 2% PVP-40, 1.4M NaCl, 100 mM Tris-HCl (pH 8.0) and 25 mM EDTA (pH 8.0). Subsequently, the extraction buffer was preheated to 65°C and added to the macerated material along with β -mercaptoethanol (2%). Then, the material was incubated at 65°C for 20 min. The mixture was homogenized and extracted with chloroform: isoamyl alcohol (24: 1; v/v). Extraction was followed by centrifugation at 9000 g for 15 min at 4°C. The supernatant was collected into new tubes, to which 1/3 of the volume was added with 8M LiCl. The resulting mixture was allowed to stand for 24 h at 4°C. The final precipitate was washed with 75% ethanol and resuspended in 20 μ l of autoclaved diethylpyrocarbonate-treated water. The DNA removal treatment was performed with DNase I (Invitrogen) according to the manufacturer's recommendations. Total RNA concentration and physical integrity were verified using NanoDrop™ 2000 (Thermo Scientific™) and 1.2% w/v agarose gel. The synthesis of the first cDNA strand was performed using the ImProm kit-II (Promega), according to the manufacturer's instructions.

Due to the lack of specific primers for the studied genes, primer design was carried out based on the genome of *Arabidopsis thaliana*, as follows: the sequences of the ACA11 and MCA1 proteins were obtained using the Uniprot platform (<https://www.uniprot.org/>) and, from each one, an alignment (*blast*) was performed with the *Eucalyptus grandis* proteome, on the Phytozome platform (<https://phytozome-next.jgi.doe.gov/>). The corresponding *E. grandis* nucleotide sequence was obtained from the protein sequence with the highest similarity. This sequence was used as a template for primer design through the Primer Blast tool of the NCBI platform (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The following set of primers were used: 5' – CAGCCTGTGAGACAATGGGT – 3' (forward) and 5' – TGCCTGCAAAGGATGGCT- 3' (reverse) for ACA11 and 5'- TCGGAGGTCGTACATCTTG – 3' (forward) and 5' TAATGAGCGGGACGAGCTTC 3' (reverse) for MCA1. PCR reactions were performed using a StepOnePlus™ Real-Time PCR system (Applied Biosystems) and iTaq™ Universal SYBR® Green Supermix (Promega). The Fast option was used at ramp speed and fluorescence was measured at 60°C for 40 cycles. For this analysis, the gene that encodes proteins containing the SAND domain (SAND) was used as a reference gene for the normalization of data in all samples (Martins et al. 2018). Relative expression levels were calculated using the $2^{-\Delta CT}$ method (Livak and Schmittgen 2001).

2.6 Statistical analysis

The data were submitted to a variance analysis (ANOVA) and the means were compared using Tukey's test ($p < 0.05$). In order to test the ANOVA assumptions for residual normality and homogeneity, the Shapiro-Wilk and Barlett tests were applied. Pearson correlations were used to evaluate the relationship between dependent variables (Ca concentration and accumulation, predawn leaf water potential,

photosynthesis, morphological characteristics of the plants and gene expression of *ACA11* and *MCA1*). All statistical analyzes were performed using the R statistical software version 4.0.0 (R Core 2020).

Results

Water restriction promoted the reduction of the predawn leaf water potential (Ψ_w) in all treatments. In the WR-Ca treatment, Ψ_w was lower (-0.82 MPa), while in WR + Ca treatment, Ψ_w was higher (-0.68 MPa) (Fig. 1).

Photosynthetic performance was negatively affected in WR plants, especially in -Ca treatments. Drought led to decreases of approximately 23% in the net photosynthesis rate (A) in the absence of Ca and around 9% in its presence, compared to their respective WC controls (Fig. 2a). The transpiration rate (E) decreased 61% in the absence of Ca and 20% in the presence of Ca in plants grown under water restriction in comparison with their respective controls (Fig. 2b). The imposition of water restriction promoted decreases in the values of stomatal conductance (g_s) and in the C_i/C_a ratio in all plants submitted to this condition.

WR plants had higher Ca concentration and accumulation in their roots than WC plants (Fig. 3). In plants growing in the presence of Ca under water restriction, there was an increase of 42.74% in the concentration and 73.12% in the accumulation of Ca in the roots in relation to their respective controls (WC treatment). In the plants cultivated in the absence of Ca, there was an increase of 124.43% in the concentration and 78.30% in the accumulation of Ca in the roots in relation to their respective controls (WC treatment) (Fig. 3a, b). Ca concentration and accumulation in shoots and roots were higher in plants growing in the presence of this nutrient (Fig. 3a, b). The translocation efficiency (TE) decreased with the imposition of water restriction, regardless of Ca presence or absence (Fig. 3c).

Root dry mass did not change with the imposition of water restriction on -Ca plants, but in the + Ca plants an increase was observed. Both leaf area and shoot dry mass decreased under WR treatment. However, plants growing in the presence of Ca showed higher leaf area and shoot dry mass when compared to plants from -Ca treatment, regardless of the water condition (Table 1). The root-shoot ratio was higher in plants submitted to water restriction; the increase was approximately 42% in the -Ca treatment, while it was approximately 72% in the + Ca treatment. The presence of Ca enabled an increase in the root-shoot ratio in all plants when compared to plants in the absence of Ca (Table 1). Characteristics related to root morphology, such as root volume, total root length and root surface area did not differ between WC and WR plants, and values were always higher in the presence of Ca. The average root diameter did not differ between treatments (Table 1).

Table 1

Morphological characteristics of a drought tolerant eucalyptus clone (VM01) plants grown in hydroponic solution in the presence or absence of Ca, with or without water restriction.

	- Ca		+ Ca	
	Water comfort	Water restriction	Water comfort	Water restriction
Leaf area (cm ²)	824.39 ± 193.5 Ab	299.01 ± 71.17 Bb	1188.52 ± 132.53 Aa	514.83 ± 57.79 Ba
Shoot dry mass weight (g plant ⁻¹)	5.16 ± 1.13 Ab	3.09 ± 0.65 Bb	6.44 ± 1.15 Aa	5.06 ± 0.60 Ba
Root dry mass weight (g plant ⁻¹)	1.40 ± 0.14 Ab	1.17 ± 0.25 Ab	1.75 ± 0.20 Ba	2.09 ± 0.17 Aa
Root-shoot ratio	0.19 ± 0.04 Bb	0.45 ± 0.09 Ab	0.49 ± 0.07 Ba	0.68 ± 0.15 Aa
Root volume (cm ³)	4.85 ± 1.01 Ab	6.33 ± 1.37 Ab	8.47 ± 1.38 Aa	9.02 ± 0.92 Aa
Total root length (cm)	15587.10 ± 3707.30 Ab	22925.47 ± 8490.34 Ab	26649.03 ± 4352.99 Aa	33406.12 ± 5227.57 Aa
Root surface area (cm ²)	973.64 ± 216.00 Ab	1328.31 ± 391.10 Ab	1681.66 ± 272.00 Aa	1938.10 ± 247.53 Aa
Average root diameter (mm)	0.20 ± 0.01 Aa	0.21 ± 0.02 Aa	0.20 ± 0.01 Aa	0.19 ± 0.01 Aa

*Different capital letters indicate a significant water condition effect (presence or absence of water restriction) with the same calcium supply. Lowercase letters indicate significant differences between calcium supply with the same water condition ($p \leq 0.05$, Tukey's test). Means ± standard error.

The expression of Ca influx coding genes varied according to water condition and Ca supply. The *ACA11* gene expression was higher in the roots of WR plants in relation to WC plants (Fig. 4a). On the other hand, the expression of the *MCA1* gene in the roots was lower in plants under water restriction (Fig. 4b). Both genes presented higher expression in the roots of plants grown in the absence of Ca in WC and WR treatments (Fig. 4).

Ca concentration and accumulation in shoots and roots were positively correlated with the morphological characteristics of the root (length, area, surface and volume). Ca concentration and accumulation in roots showed a positive correlation with the root/shoot ratio and a negative correlation with the translocation efficiency. In addition, translocation efficiency was negatively correlated with the root/shoot ratio. Ca concentration in shoots and roots presented negative correlations with the expression of the *MCA1* gene. A negative correlation was also observed between *MCA1* expression and total root volume, total root surface, root length and root/shoot ratio. The *ACA11* gene expression was negatively correlated with leaf area, shoot Ca accumulation, net photosynthesis rate and transpiration (Fig. 5).

Discussion

Our results revealed that eucalyptus plants cultivated under water restriction presented a higher expression of *ACA11* and a lower expression of *MCA1* as well as Ca concentration and accumulation in the roots compared to plants in the treatment grown under water sufficient conditions. *ACA11* expression occurs in the large vacuoles of root epidermal cells, including root hairs, where it plays an important role in the growth of these cells and Ca storage (Lee et al. 2007; García Bossi et al. 2020). Our results indicate that an increased Ca concentration and accumulation in the roots of the VM01 clone plants under drought conditions is associated with greater expression of the *ACA11* gene and, consequently, greater influx and accumulation of this nutrient in the vacuoles of root cells. On the other hand, we found a lower expression of the *MCA1* in plants submitted to water restriction when compared to plants with adequate water supply. *MCA1* is expressed in the plasma membrane of endoderm cells and in the stele of primary roots (Yamanaka et al. 2010). *MCA1* protein is likely to be involved in the uptake and transport of Ca into the xylem via the symplastic pathway in endodermal and vascular cells (Nakagawa et al. 2007; Yamanaka et al. 2010). The relative contribution of alloplastic and symplastic pathways in the displacement of Ca to the xylem is unknown and appears to be species-dependent (White 2000; White and Broadley 2003b; Cholewa and Peterson 2004; González-Fontes et al. 2017). However, the presence of Kasparian strips around the endoderm restricts Ca movement through the apoplastic pathway. It seems reasonable to suggest that the lower expression of *MCA1* in the roots of VM01 eucalyptus clone under water-restricted conditions decreases the influx into the endoderm and, consequently, the loading of Ca into the xylem (Fig. 6). In agreement, we found increased Ca concentration and accumulation in roots of water stressed plants and a negative correlation of root Ca and *MCA1* expression.

We found that Ca supply increased A , E , g_s and C_i/C_a in water stressed plants. Water restriction and Ca increased root growth and had opposite effects on Ψ_w . Water stressed plants had lower Ψ_w and this effect was reduced in the presence of Ca as reported for other plant species (Chari et al. 1986; Xu et al. 2013; Rezayian et al. 2018; Khushboo et al. 2018; Xue et al. 2018; Leng et al. 2020). Ca also increased plant root-to-shoot ratio, one of the main responses of drought-tolerant *Eucalyptus* species to low water availability (Maseda and Fernández 2016; Saadaoui et al. 2017; Müller et al. 2020). Our results are also in agreement with previous studies on *E. camaldulensis*. Hakamada et al (2020) showed that plants from a eucalyptus clone obtained from *E. camaldulensis*, cultivated under field conditions, allocated more photoassimilates to the roots in an attempt to avoid the negative effects of drought. In addition, *E. camaldulensis* hybrids selected from dry regions are usually characterized by larger amounts of thin roots and greater root system lengths (Pinheiro et al. 2016).

Conclusions

An optimal Ca nutrition reduces multiple negative effects of water restriction in plants. In this study, we found that water restriction may reduce the expression of *MCA1* and increase *ACA11* contributing to an increased Ca concentration and accumulation roots of drought-tolerant eucalypt plants. However, it is important to note that caution must be taken when interpreting the results described here since

experimental validation of the involvement of the transporters encoded by these genes is still required. This fact aside, our findings bring novel evidence of an important yet unrecognised mechanism that orchestrate Ca homeostasis in a drought-tolerant tree genotype, paving the way to development of productive eucalyptus materials under water limiting conditions. Our results therefore contribute to explain, at least partially, several controversial points regarding drought tolerance in eucalyptus plants. Notwithstanding, future experiments with different woody species and genetic materials are necessary to strengthen and expand the evidence observed in this study.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

P.R.B: experiment conduction; data collection; data analysis and writing. H.O.O: data collection; data analysis and reading the final draft. W.L.A: experiment planning and reading the final draft. S.V.V, A.P.B and N.F.B: experiment planning, guidance for data collection and analysis, writing-review. All authors read and approved the final version of the manuscript.

Data availability

All data generated or analysed during this study are included in this published article.

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Figures

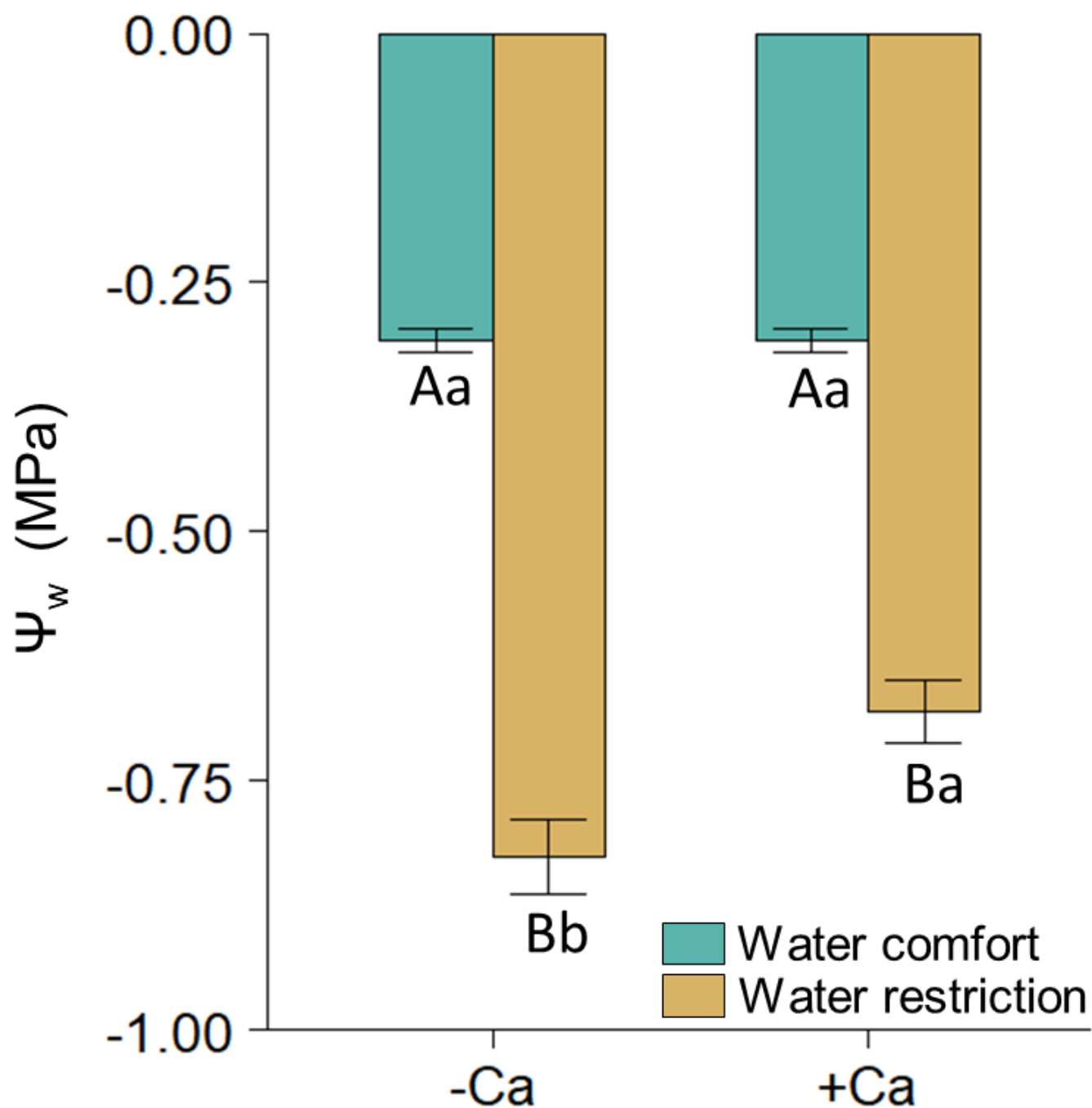


Figure 1

Predawn leaf water potential (Ψ_w) of VM01 eucalyptus clone cultivated in hydroponic solution in the presence (+Ca) or absence (-Ca) of Ca, with and without water restriction. Different capital letters indicate a significant water condition effect (water comfort and water restriction) with the same Ca supply. Lowercase letters indicate significant differences between calcium supply with the same water condition ($p \leq 0.05$, Tukey's test). Bars represent the mean $\pm$ standard error.

Water comfort
Water restriction

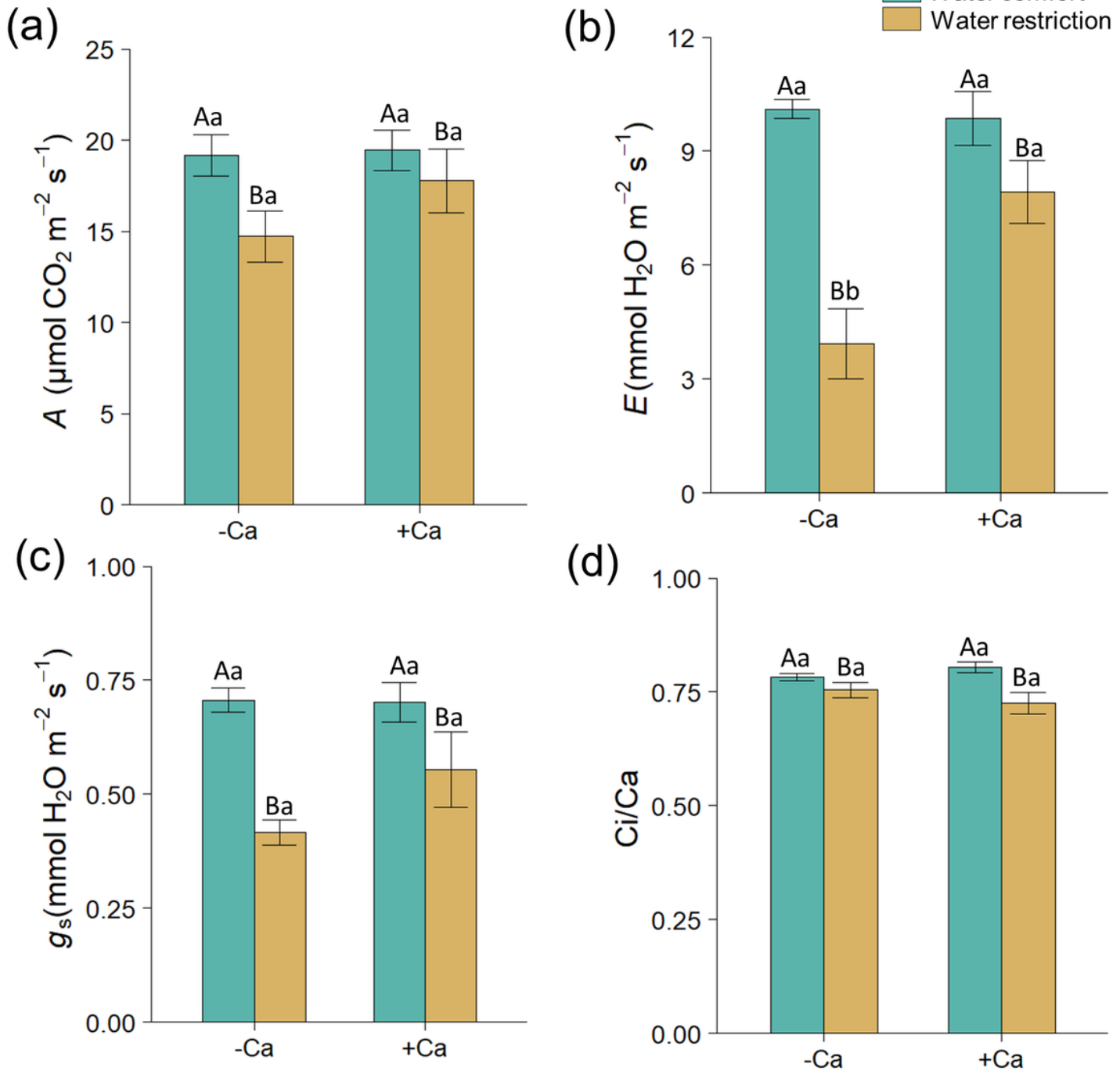


Figure 2

(a) CO_2 net assimilation rate (A), (b) Transpiration rate (E), (c) stomatal conductance (g_s) and (d) ratio between internal and external CO_2 concentration in VM01 eucalyptus clone cultivated in hydroponic solution in the presence (+Ca) or absence (-Ca) of Ca, with or without water restriction. Different capital letters indicate a significant water condition effect (water comfort and water restriction) with the same Ca supply. Lowercase letters indicate significant differences between calcium supply with the same water condition ($p \leq 0.05$, Tukey's test). Bars represent the mean $\pm$ standard error.

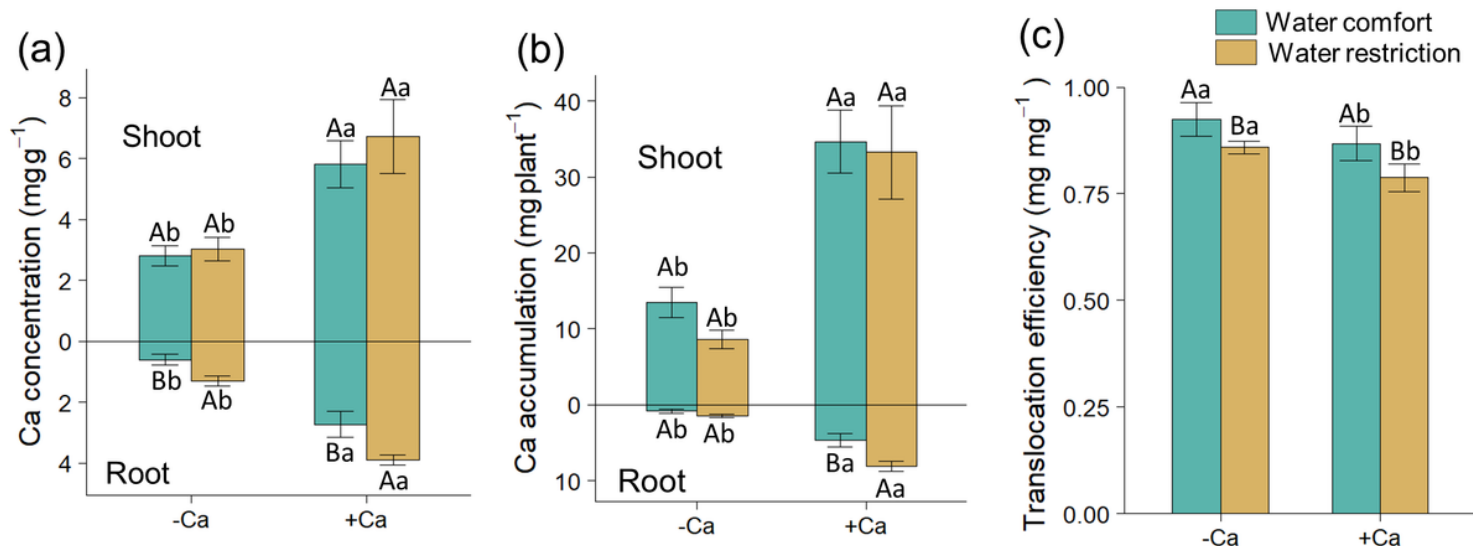


Figure 3

(a) Ca concentration in shoots and roots, (b) Ca accumulation in shoots and roots and (c) Ca translocation efficiency in VM01 eucalyptus clone grown in hydroponic solution in the presence (+Ca) or absence (-Ca) of Ca, with or without water restriction. Different capital letters indicate a significant water condition effect (water comfort and water restriction) with the same Ca supply. Lowercase letters indicate significant differences between Ca supply with the same water condition ($p \leq 0.05$, Tukey's test). Bars represent the mean $\pm$ standard error.

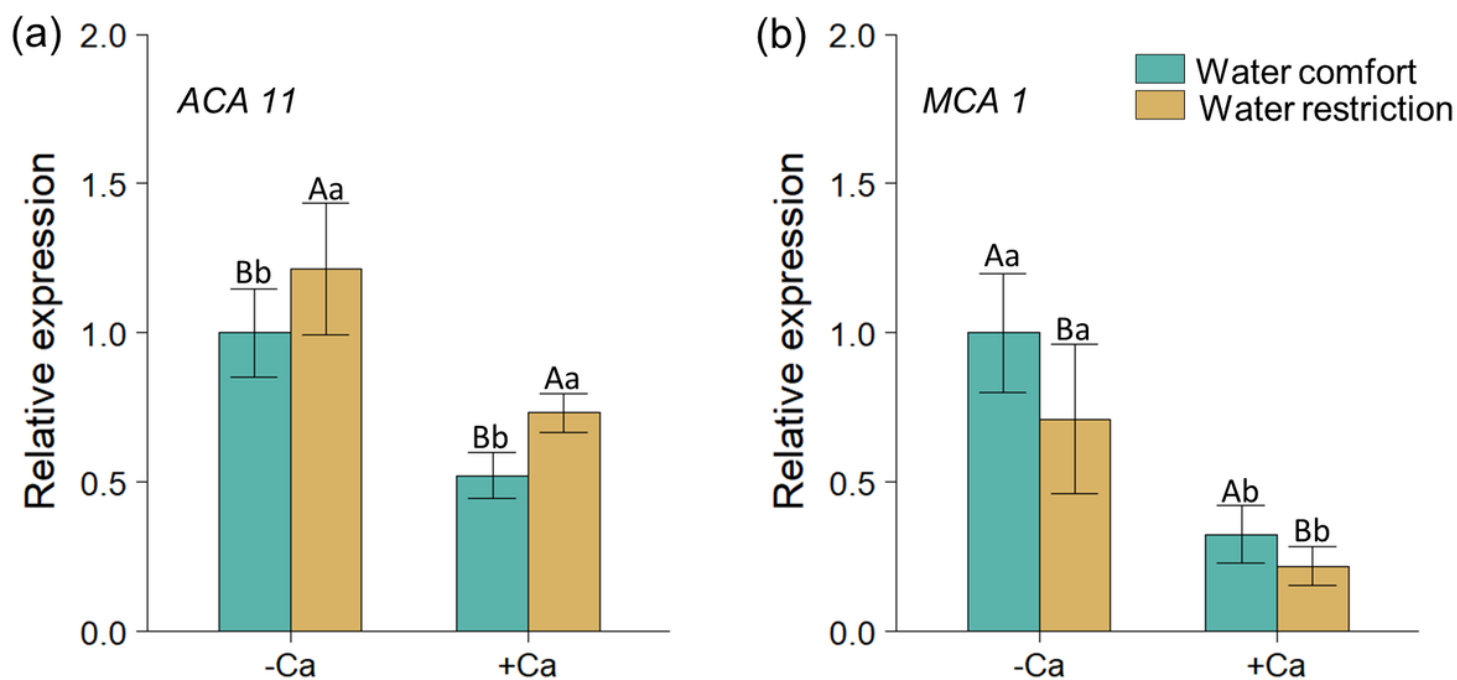


Figure 4

(a) Relative expression of genes *ACA11* that codes an inflowing Ca transporter in the tonoplast and (b) *MCA1* that codes an inflowing Ca transporter located in the plasma membrane of the endoderm in roots of VM01 eucalyptus clone grown in hydroponic solution in the presence (+Ca) or absence (-Ca) of Ca, with or without water restriction. Different capital letters indicate a significant water condition effect (water comfort and water restriction) with the same Ca supply. Lowercase letters indicate significant differences between calcium supply with the same water condition ($p \leq 0.05$, Tukey's test). Bars represent the mean $\pm$ standard error.

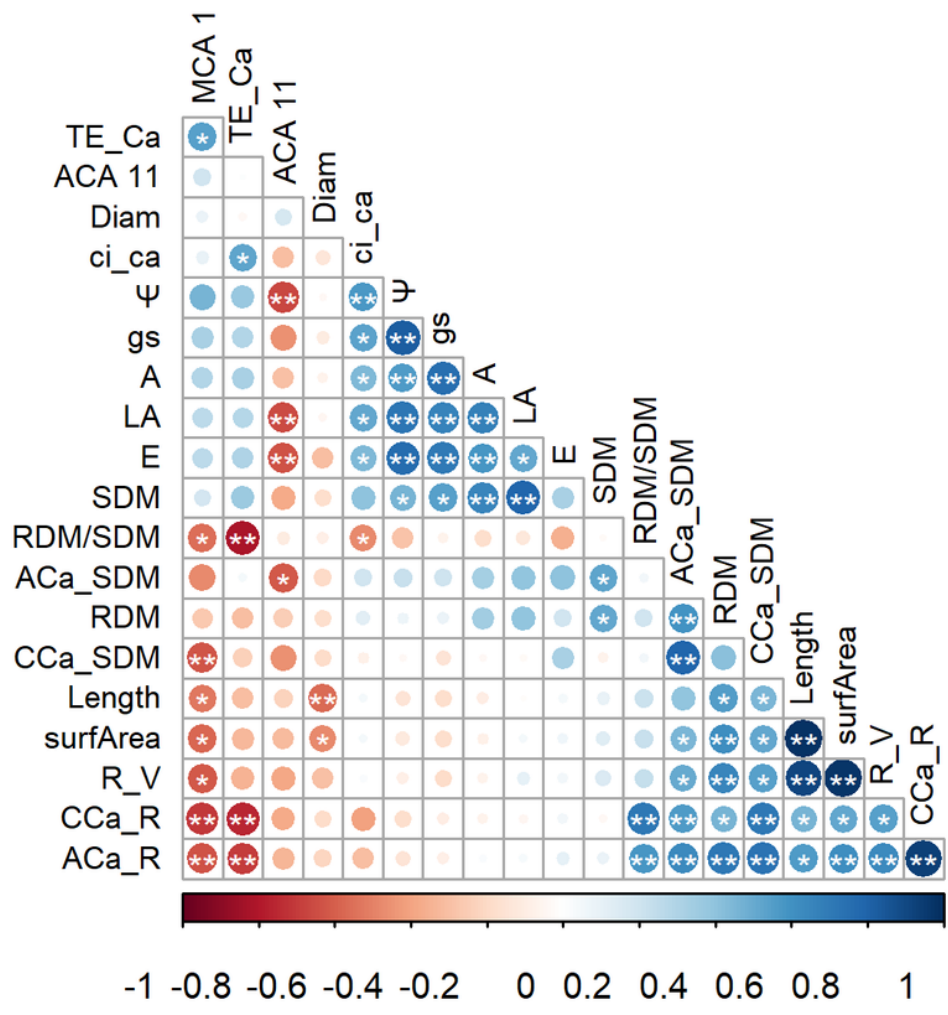


Figure 5

Pearson correlation matrix between calcium concentration /accumulation, photosynthesis, morphological characteristics and Ca transporters. *MCA1*, Ca transporters in the plasma membrane of the endoderm; *ACA11*, vacuole Ca transporter; A, net photosynthesis rate; E, transpiration; gs, stomatal conductance; ci_ca, ratio between internal and external CO₂ concentration, TE_Ca, Ca translocation efficiency; Diam, mean root diameter; LA, leaf area, SDM, shoot dry mass; RDM, root dry mass; RDM/SDM, root/shoot ratio; Length, root length; surfArea, root surface area; R_V, total root volume; CCa_SDM, Ca concentration in the

shoot, CCa_R, Ca concentration in the root; ACa_SDM, Ca accumulation in the shoot; ACa_R Ca accumulation in the root; Significant correlations ** ($p < 0.01$) and * ($p < 0.05$).

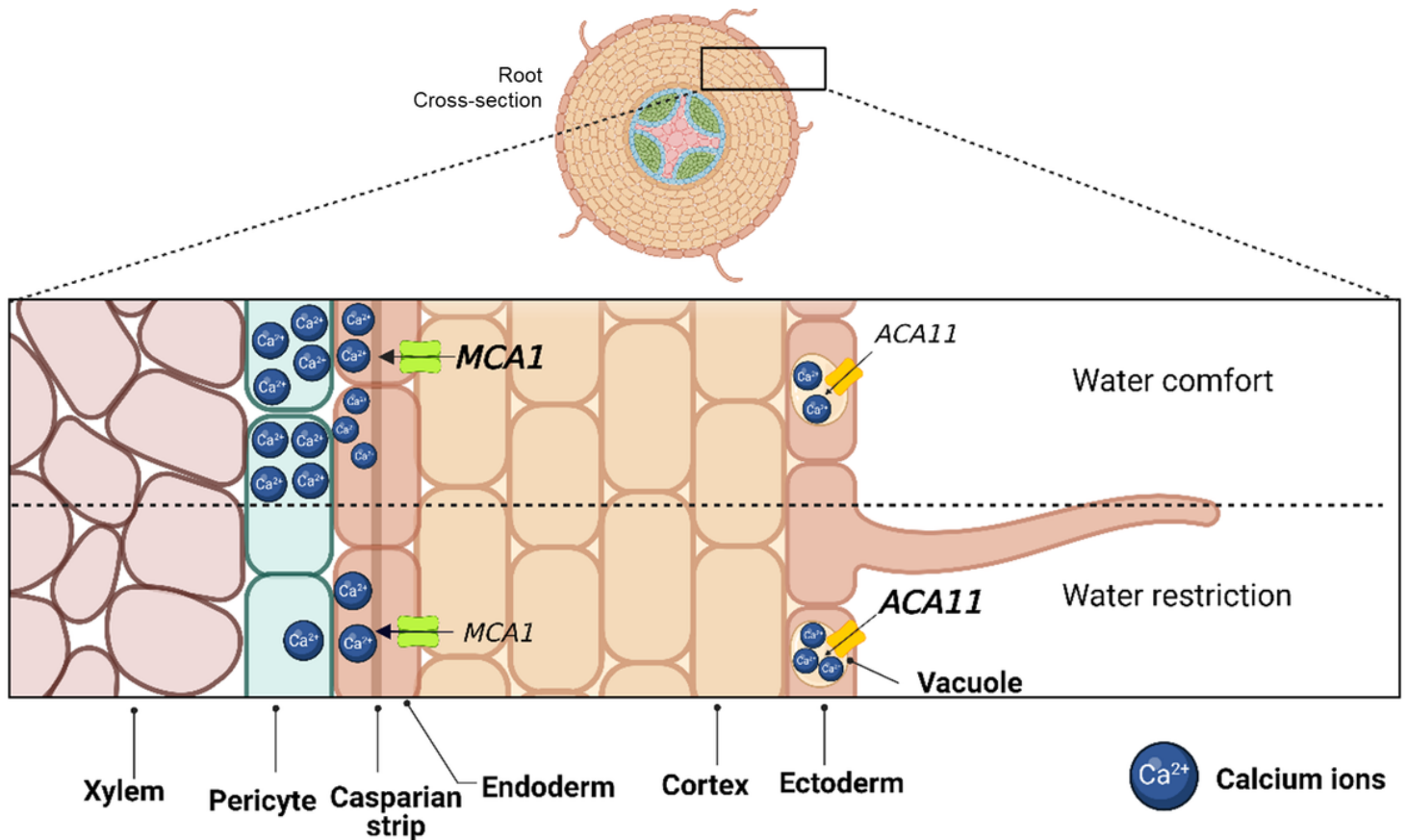


Figure 6

Model for the regulation of the genes *ACA11* and *MCA1*. *ACA11* codes the Ca influx transporter in the tonoplast. *MCA1* codes the Ca influx transporter located in the plasma membrane of the endoderm. The scheme illustrates results obtained in VM01 eucalyptus clone cultivated in hydroponic solution in the presence or absence of water restriction. Briefly, under water restriction, eucalyptus plants displayed a higher expression of *ACA11* coupled with a lower expression of *MCA1* and a reduced Ca concentration and accumulation in the roots compared to plants under water sufficient conditions. Text font size changes represent variations in the expression of the *ACA11* and *MCA1* genes (larger font, higher expression). (Created with BioRender.com).