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Element content and expression of genes of interest in guard cells are connected to spatiotemporal variations in stomatal conductance

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Abstract

Element content and expression of genes of interest on single cell types, such as stomata, provide valuable insights into their specific physiology, improving our understanding of leaf gas exchange regulation. We investigated how far differences in stomatal conductance (g_s) can be ascribed to changes in guard cells functioning in amphistomateous leaves. g_s was measured during the day on both leaf sides, on well-watered and drought-stressed trees (two *Populus euramericana* Moench and two *Populus nigra* L. genotypes). In parallel, guard cells were dissected for element content and gene expressions analyses. Both were strongly arranged according to genotype, and drought had the lowest impact overall. Normalizing the data by genotype highlighted a structure on the basis of leaf sides and time of day both for element content and gene expression. Guard cells magnesium, phosphorus, and chlorine were the most abundant on the abaxial side in the morning, where g_s was at the highest. In contrast, genes encoding H^+-ATPase and aquaporins were usually more abundant in the afternoon, whereas genes encoding Ca^{2+}-vacuolar antiporters, K^+ channels, and ABA-related genes were in general more abundant on the adaxial side. Our work highlights the unique physiology of each leaf side and their analogous rhythmicity through the day.

KEYWORDS

abaxial and adaxial surfaces, droughts, elements, gene expression, plant stomata, Populus, stomatal conductance

1 | INTRODUCTION

Stomata are small pores on the surface of the leaf responsible for almost all gas exchange between the leaf internal environment and the surrounding air. They are composed of two guard cells that modify their volume, adjusting the central aperture and controlling the flow of gases in and out of the leaf. These adjustments are regulated by a variety of internal and external factors, including but not limited to water availability (Monclus et al., 2006), irradiance (Shimazaki, Doi, Assmann, & Kinoshita, 2007), and circadian rhythms (de Dios, 2017). Regulation of stomatal aperture is considered a key phenomenon, maximizing CO_2 uptake for photosynthesis with regard to water loss by transpiration (Cowan & Farquhar, 1977), whose importance increases as internal and environmental conditions become more limiting.

Poplars are dicots, model trees for molecular studies (Jansson & Douglas, 2007; Tuskan et al., 2006), with amphistomateous leaves. As such, despite being in the same environment, each leaf side experiences a different magnitude of irradiance and temperature (Clum,

1926; Sheriff, 1979). Literature evidence suggests that guard cell sensitivity to irradiance (Kassam, 1973), vapour-pressure deficit (Pallardy & Kozlowski, 1979), leaf water potential (Kanemasu & Tanner, 1969), and ozone (Dumont et al., 2014) differs between leaf sides. Moreover, some species showed independent stomatal regulation between surfaces (Mott, 2007; Richardson, Brodribb, & Jordan, 2017), consistent with an optimal stomatal behaviour of each leaf side. This suggests that, in poplars, stomatal movements under drought and along the day may depend on the side of the leaf.

As a consequence of global climate change, droughts are expected to be more intense and frequent in the future (Dai, 2012; Intergovernmental Panel on Climate Change, 2014). A prolonged reduction in water availability will induce a wide variety of severe and long-lasting humanitarian, economic, and environmental impacts on ecosystems and societies (Touma, Ashfaq, Nayak, Kao, & Diffenbaugh, 2015). Among them, the impact of drought on forests is a central concern in global climate change research given the interrelation between forests and climate (Bonan, 2008). Poplars are commonly distributed along riparian ecosystems due to their high water requirements (Tschaplinski & Blake, 1989). Their cultivation however usually extend to mesophyte habitats where they are more frequently subjected to soil water deficit. Plant response to drought is complex, but stomatal closure and a reduction in plant productivity is generally observed (Ciais et al., 2005; Coopman et al., 2008; Monclus et al., 2006).

For soil water deficit and high vapor pressure deficit (VPD) conditions, it remains unresolved whether hydraulic or hormonal mechanisms are responsible for stomatal closure (Merilo et al., 2018; Rodriguez-Dominguez et al., 2016). One hypothesis states that a decrease in stomatal conductance (g_s) during drought is mostly driven by hormonal signals such as abscisic acid (ABA), which are generated independently of leaf water status (Davies & Zhang, 1991; Dodd, 2005; Tardieu & Simonneau, 1998). However, the complex regulation of stomatal functioning can involve a number of actors, from phytohormones to strigolactones (Acharya & Assmann, 2009; Lv et al., 2018). It has alternatively been proposed that stomatal closure during edaphic droughts is caused by an actively mediated negative feedback response of stomata to reduced leaf turgor (Brodribb & Cochard, 2009; Buckley, 2005; Sperry, Hacke, Oren, & Comstock, 2002). ABA is a central plant hormone responsible for promoting stomatal closure and inhibiting opening under drought (Chaves, Maroco, & Pereira, 2003; Huang, Wu, Abrams, & Cutler, 2008). In recent years, evidence has accumulated showing that guard cells possess the full ABA biosynthesis pathway (Bauer et al., 2013) and the early ABA signal transduction (Zhang et al., 2015). ABA binds and inhibits PP2C-type phosphatases by binding to the PYR/PYLs family of proteins (Merilo et al., 2013), including ABI1 (Park et al., 2009; Yoshida et al., 2006). This in turns relieves the repression on OST1, which inactivates the KAT1 inward-rectifying potassium channel (Sato et al., 2009) and activates the S-type anion channel SLAC1 (Geiger et al., 2009). These channels, among others, induce a reduction of osmotic potential inside the guard cells leading to a flow of water outward, which results in stomatal closure (Schroeder & Hedrich, 1989; Schroeder, Raschke, & Neher, 1987).

In addition, circadian rhythms in transcript accumulation (Wilkins, Waldron, Nahal, Provart, & Campbell, 2009) and gas exchange (de Dios, 2017) are limiting our ability to fully understand the molecular signals involved in stomatal movements. A number of genes involved in the regulation of stomatal movements were found to be gated by the circadian clock, including ABA biosynthesis and signalling pathways (for a complete review, see Seung, Risopatron, Jones, & Marc, 2012) and *TIPs* and *PIPs* (aquaporins) mRNA levels (Chaumont & Tyerman, 2014). Circadian rhythms appear to be cell-specific as stomata were shown to have a distinct rhythmicity compared with surrounding mesophyll cells in *Arabidopsis thaliana* (Hassidim et al., 2017; Yakir et al., 2011). Given the importance of stomata in regulating gas exchange and thus the productivity of plantations, there is a need to better understand the regulation of g_s during the day and under different water availabilities.

Specifically studying stomata independently from the surrounding mesophyll cells is not trivial (Leonhardt et al., 2004). Hence, not much is known about the physiological and molecular processes in guard cells driving stomatal conductance in trees species such as poplars. To increase our understanding of the differences in such cellular processes among genotypes, between leaf sides, during the day and plastic responses related to soil water deficit, there is a need for studies targeting a specific cell type.

To test well-established processes exerted by the guard cells, quantification of their element content and their expression of 27 genes of interest were monitored separately on both leaf sides from leaves harvested in the morning and the afternoon. We used two *Populus nigra* × *Populus deltoides* (*Populus euramericana*, Moench) and two *P. nigra* (L.) genotypes grown in parallel under control and soil water deficit conditions. Given this experimental set-up, we aimed to answer the following questions: (a) Is there a constitutive difference among genotypes of element content and gene expression in poplar guard cells? (b) Do factors such as water regime, time of the day, and leaf side induce a plastic response in terms of guard cells (i) element content and (ii) gene expression?

2 | MATERIAL AND METHOD

2.1 | Plant material and growth conditions

Woody cuttings of four poplar genotypes belonging to different species or commercial hybrids (Carpaccio and I214: *P. deltoides* × *P. nigra*, Moench, 6J29 and N38: *P. nigra*, L.) were grown in a fully automated glasshouse under natural light with a free range for air temperature between 15°C and 25°C at INRA-Grand-Est (48°45′09.3″N, 6°20′27.6″E; Champenoux, France), as described previously (Durand, Brendel, Buré, & Le Thiec, 2019). Genotypes were chosen based on their contrasting drought response: I214 and 6J29 are generally found to be sensitive to drought (Chen, Wang, Altman, & Huttermann, 1997; Giovannelli et al., 2007; Muller & Lambs, 2009; Viger et al., 2016). Sixteen cuttings per genotype were planted in 9.5 kg (±1.5%) of a sand/peat mixture (1/1, v/v), complemented with fertilizer (1 g L^{-1} of

$CaMg(CO_3)_2$ and 20 g of Nutricote T100, 13:13:13 NPK and micronutrients, FERTIL S.A.S., Boulogne-Billancourt, France). Each 10-L pot was covered with 1.4 kg of white marble gravel (8–12 mm). Pots were weighed and watered to 27.9% volumetric soil water content (SWC; 85% of field capacity) at least three times a day with a robot (Durand et al., 2019). After 60 days of precultivation, watering was stopped for half the individuals (randomly chosen) until an SWC of 18.3% was reached. This controlled soil water deficit was maintained constant for 25 days using a pot-specific linear regression between SWC measured by time domain reflectometry (HD2, IMKO, Ettlingen, Germany) and pot mass ($R^2 > .8$). Reference masses were adjusted each week to take the plant growth into account. Growth, transpiration, and meteorological data are available in Durand et al. (2019).

2.2 | Leaf gas exchange and leaf sampling

Leaf gas exchange and leaf sampling were performed sequentially in order to accurately capture stomatal conductance at the time of sampling. The first fully expanded leaf (selected before the drought) and the one immediately below were sampled 21 days after the start of the drought experiment at 10:30 a.m. and 3:00 p.m. local time, respectively. At the time, the natural daytime lasted 16 hr (starting at 4:30), global radiation was 297.2 ± 47.8 and 145.6 ± 45.1 W m^{-2}, air temperature was 24.9 ± 0.2 and 24.8 ± 0.2°C, and vapour-pressure deficit was 1.31 ± 0.02 and 1.20 ± 0.07 kPa for the two sampling events, respectively. From previous g_s time-course measurements, stomata were likely fully open in the morning and in a closing phase in the afternoon (Durand et al., 2019). The last watering event was completed 2 hr before each sampling. A leaf porometer was used in order to measure g_s separately on the abaxial and adaxial surfaces of the leaves (SC-1 porometer; Decagon Devices, Inc., Pullman, WA, USA). Stomata being on the leaf surfaces, sampled leaves were flash frozen in liquid nitrogen immediately after measurements, to guarantee both RNA integrity and that diffusive elements remained in guard cells (Amsellem et al., 1983; Jaiprakash et al., 2003).

2.3 | X-ray microanalysis

Sample preparation and microanalyses were performed following Dumont et al. (2014), except for the use of a tungsten coating on the samples and a probe intensity of 1 nA, in order to allow analyses in stomata specifically. Although the analysed X-rays originate from a lower layer in the sample than electrons (Amsellem et al., 1983), the electron beam diameter and depth of penetration is lower than 2 μm and has been simulated in a Monte Carlo procedure (Pouchou & Pichoir, 1991); 20 guard cells, each belonging to different stomata of freeze-dried leaves, were specifically targeted and analysed for each of the two leaf sides, the two times of day, the two water treatments, and the four genotypes with six replicates in each condition. Microanalyses were corrected by measuring the content of beech leaves powder of known mass fraction (CRM 100, BCR® certified reference material, Commission of the European Communities, Brussels, Belgium) for sodium (Na), magnesium (Mg), phosphorus (P), chlorine (Cl), potassium (K), and calcium (Ca). All forms were measured, both organic and inorganic. We also computed a ratio of anions over cations for each guard cell (i.e., P and Cl over Na, Mg, K, and Ca later referred to as *An/Cat*). Values were converted from percent to "per mille" (or mg g^{-1}) of dry mass.

2.4 | Guard cell microdissection, RNA extraction and cDNA amplification

Guard cells microdissection, RNA isolation, RNA reverse-transcription, and cDNA amplification were performed following Dumont et al. (2014). A brief description of the methods is given hereinafter. If not otherwise specified, protocols were performed following the manufacturer's instructions; 1,000 stomata per sample were microdissected using the PALM MicroBeam system (Carl Zeiss MicroImaging GmbH, Jena, Germany). Total RNA was extracted using Qiagen RNeasy Plus Micro Kit (Qiagen, Courtaboeuf, France). Previous tests in our lab and in the literature show that the process of freeze-drying and preparing microscopic slide does not impact the RNA integrity (Damsteegt, McHugh, & Lokman, 2016; Dumont et al., 2014; García-Baldenegro et al., 2015; Heinen et al., 2014; Jaiprakash et al., 2003). Transplex® Whole Transcriptome Amplification Kit (MERCK, Saint-Quentin Fallavier, France) was used for RNA reverse transcription and cDNA amplification (100 μl twice by sample). The latter was purified by Gene Elute PCR (SIGMA, Saint-Quentin Fallavier, France). cDNA purity and concentration were measured (Nanodrop 1000; Thermo Scientific, Illkirch, France). All cDNA samples exhibited a good purity level ($1.6 < A_{260}/A_{280} < 2.0$ and $1.3 < A_{260}/A_{230} < 1.9$), and each sample was diluted to get 2.5 ng μl^{-1}. Absence of genomic DNA was confirmed by PCR with intron-flanking primers.

2.5 | Expression of genes of interest and real-time PCR

The genes of interest were retrieved on the Populus genome v3.0 (Phytozome, RRID:SCR_006507, https://phytozome.jgi.doe.gov/pz/portal.html; Tuskan et al., 2006). The genes of interest were selected for their putative role in encoding ionic pumps, aquaporins, transporters involved in stomatal movements, and phototropins, β-carbonic anhydrases, and genes linked to ABA biosynthesis and signalling. Genes were selected in regard to previous studies (Cohen et al., 2010; Dumont et al., 2014; Heinen et al., 2014) on the basis of a high expression level in poplar guard cells (when available, and in leaves otherwise), their differential expression among genotypes, between leaf sides, water availability, and time of day. The design of primers, with adequate specificity for all four genotypes, was also a limiting step for gene selection. Only primers with a reduced risk of primer dimer formation or nonspecific amplification were selected. Because the expression of gene of interest approach is not exhaustive by definition, we chose to investigate genes with different roles, instead of a whole family of genes. Thus, our interpretation is not limited to a

single family of transporters but benefits from a more exhaustive assessment of guard cell processes linked with stomatal regulation. The targeted genes and their description are provided in Table 1 (additional information is given in Table S1).

Real-time PCR was performed in a 96-well Mx3005P thermocycler (Agilent, Waghaeusel-Wiesental, Germany) using the recommended cycling programme: 5 min at 95°C, 40 cycles of 5 s at 95°C, and 20 s at 58°C or 60°C depending on primers, followed by melting cycle to ensure a single amplicon (Dumont et al., 2014). The mix contained 10 ng of cDNA, the reference dye (ROX, Agilent technologies), gene-specific primers, and the Brilliant III UltraFast SYBR GREEN qPCR Master Mix (Agilent, Santa Clara, CA). No-template control reactions were prepared for each gene. The plate set-up included all samples from two genotypes and the negative controls. The Ct values were determined with the same threshold. For each targeted gene-genotype group, efficiency was calculated from standard curves over seven dilutions. Efficiencies varied from 65% to 102% ($R^2 \geq .985$) and were taken into account in all subsequent calculations (Bizet et al., 2015). Among the studied genes, *UBQ11* (Brunner, Yakovlev, & Strauss, 2004), *QUAC1* (Dumont et al., 2014), and *BAM1* were the most stable and used as references, together with an external control (GENORM v3.5, RRID:SCR_006763, https://genorm.cmgg.be/; Mestdagh et al., 2009; Vandesompele et al., 2002). The fluorescent threshold being set constant, the nonnormalized expression level was computed as $RQ = 1/(Ta \cdot E^{Ct})$, Ta being the amplicon length in bp and E the efficiency (Bizet et al., 2015; Gutierrez et al., 2008).

TABLE 1 Description of the genes studied and their localization

Genes	Description
NCED3.1	Key enzyme in ABA biosynthesis (9-cis-epoxycarotenoid dioxygenase).
NCED3.2	Key enzyme in ABA biosynthesis (9-cis-epoxycarotenoid dioxygenase).
ABI1	Protein phosphatases type 2C (PP2Cs), negative regulator of ABA promotion of stomatal closure.
OST1	SNF1-related protein kinase, involved in ABA-induced stomatal closure by activating of S-type anion channels and inhibiting inward rectifying potassium channel.
PYL2	PYR/PYL/RCAR family of protein, inhibits the activity of group-A protein phosphatases type 2C (PP2Cs) when activated by ABA.
PYL4	PYR/PYL/RCAR family of protein, inhibits the activity of group-A protein phosphatases type 2C (PP2Cs) when activated by ABA.
PYL8	PYR/PYL/RCAR family of protein, inhibits the activity of group-A protein phosphatases type 2C (PP2Cs) when activated by ABA.
CA1	Beta-carbonic anhydrase, involved in CO_2-mediated stomatal closure (chloroplast).
CA4	Beta-carbonic anhydrase, involved in CO_2-mediated stomatal closure (PM).
PHOT1	Blue light photoreceptors, mediate blue light-dependent activation of the plasma membrane H^+-ATPases
PHOT2	Blue light photoreceptors, mediate blue light-dependent activation of the plasma membrane H^+-ATPases
AKT2	Inward rectifying potassium channel (PM), responsible for the Ca^{2+} sensitivity of the K^+ uptake channel.
KAT1.2	Inward rectifying potassium channel (PM), responsible for the Ca^{2+} sensitivity of the K^+ uptake channel.
KAT3	Inward rectifying potassium channel (PM), responsible for the Ca^{2+} sensitivity of the K^+ uptake channel. Involved in down-regulating AKT1 and KAT1 channel activity by forming heteromers.
OST2	Proton ATPases (PM)
AHA11	Proton ATPases (PM)
SLAC1	Slow anion channel (PM), involved in stomatal closure in response to a variety of signals including elevated CO_2, ozone, ABA, darkness, and humidity.
CAX1	Calcium antiporter (V)
CAX1.6	Calcium antiporter (V)
NHX1.13	Potassium, sodium/proton antiporter (V) involved in salt tolerance and ion homeostasis
PIP1.2	Aquaporin: plasma membrane intrinsic protein (PM)
PIP2.1	Aquaporin: plasma membrane intrinsic protein (PM)
PIP2.5	Aquaporin: plasma membrane intrinsic protein (PM)
TIP1.3	Aquaporin: tonoplast intrinsic protein (V)
TIP1.4	Aquaporin: tonoplast intrinsic protein (V)
TIP2.1	Aquaporin: tonoplast intrinsic protein (V)
TIP2.2	Aquaporin: tonoplast intrinsic protein (V)

[a]Abbreviations: PM, plasma membrane; V: vacuole.

The normalized expression of targeted genes were computed by dividing their RQ by the geometric average of the reference gene's RQ (Bizet et al., 2015).

2.6 | Statistics

Statistics were performed using R 3.5.1 (R Project for Statistical Computing, RRID:SCR_001905, https://cran.r-project.org/, all data used are available in Table S2). We used a type 2 factorial analysis of variance design to study the variation of g_s (n = 6), element content (n = 6), and normalized mRNA levels (n = 4) by genotype, water regime, time of day, and leaf side and their first-order interaction. Given the complexity of the design, we decided to focus our interpretation of the dataset on the significant main factors and their first-order interactions, instead of computing all pairwise comparisons between our 32 groups. This enabled us to explore the major trends with a larger sample size and a higher statistical power than if we specifically addressed each pairwise comparison. Normalized transcript levels were transformed with a logarithmic function of base 2 in order to achieve a normal distribution of the data. Post hoc contrast analyses were performed to test differences between specific modalities of the studied factors depending their significance, and P values were adjusted to control for the false discovery rate. The partial R^2 was calculated to compare the contribution with the explained variable of each of the four dependent variables. We performed a principal component analysis (PCA) on the six elements studied and the ratio *An/Cat* on one hand and on the expression data of 27 genes of interest on the other hand. We fitted to every variable a linear model that included the highly significant genotype effect as a dependent factor. The residuals of these models were used as the data for the two PCA in order to emphasize the other main effects (water regime, time of day, and leaf side). Stomatal conductance data were fitted with a similar model, and the residuals were added as a supplementary variable.

3 | RESULTS

3.1 | Stomatal conductance

Testing for genotypic, water availability, time of day, and leaf side effects on g_s measured immediately before sampling the leaves, we found all four to be significant (Table 2, P < .002 in all cases). Drought-stressed poplars had an overall 20% lower g_s than well-watered trees. Because there was no interaction with the water treatment, we showed the average g_s of both treatments (Figure 1). The significant interaction between genotype and time of day (P = .023) showed that the afternoon decrease of g_s tended to be stronger in both hybrid poplars than in the black poplars (Figure 1). Black poplars exhibited in general a higher g_s than the hybrid poplars, and g_s was overall higher on the abaxial side. Essentially, the highest g_s tended to be observed on the abaxial side, in the morning and on well-watered trees for all genotypes, whereas the adaxial side, in the afternoon on drought-stressed trees, showed the lowest g_s.

3.2 | Genotypic diversity of element content and gene expression within guard cells

In this study, element content was measured as the whole guard cell average. We found significant genotypic diversity in all measured elements (Figure S1, Table 2, in all cases P < .001). The genotype effect was always the factor explaining the total variance of element content the most (Table S3). To highlight these genotypic differences, we decided to show the genotype means for each element in Figure 2. Partial R^2 for genotype ranged from.16 for the P content to.7 for the Cl content (Na: .57, Mg: .49, P: .16, Cl: .70, K: 0.33, Ca: 0.21). The main elements in the guard cells were K and Ca. Na content was the highest in the guard cells of the hybrid poplars, and especially in Carpaccio were it was 3.1, 5.3, and 3.8 times higher than I214, 6J29, and N38 respectively. Additionally, Mg content was 1.7 times higher in Carpaccio than in the other three genotypes, but K content was 1.6 times higher in I214 than in the other three genotypes. On the other hand, Cl content was 4.6 times lower in both hybrid poplars than in the two black poplar genotypes. This effect was passed on in our calculation of *An/Cat*, which was 2.2 times lower in the two hybrid poplars than in the *P. nigra* genotypes. Moreover the guard cells of 6J29 showed a P and Ca content, respectively, 17% and 24% lower than in the other three genotypes on average.

The expression of genes of interest in the guard cells was also structured according to genotype. Figures 3 and S2 show the back-transformed mean of normalized mRNA levels by genotype, that is, by applying a base 2 exponential to the means of the log2-transformed data, to be consistent with statistical analyses. For all measured transcripts (in all cases P < .001) except for *CAX1* (P = .34), the genotype effect was always the factor explaining the total variance of normalized mRNA levels the most (Table S3). Partial R^2 for the genotype effect ranged from 0.13 to 0.98 for *AKT2* encoding a K^+ channel and *PYL2*, encoding an ABA receptor, respectively, with an average of 0.57. Overall, the normalized expression of *NCEDs*, encoding 9-cis-epoxycarotenoid dioxygenases involved in ABA biosynthesis, were the highest in black poplars. Specifically, N38 and 6J29 exhibited the highest accumulation of transcripts for *NCED3.1* and *NCED3.2*, respectively (Figure S2a,b). Conversely, ABA signalling pathway-related transcripts appeared more expressed in I214 guard cells, and especially the genes encoding the ABA receptor *PYL2* and *PYL8* (Figure S2e,g). Carpaccio was consistently among the genotypes with the lowest accumulation of transcripts related to both ABA biosynthesis and signalling pathways. Regarding ß-carbonic anhydrases, N38 exhibited equivalent levels of *CA1* and *CA4* mRNA, whereas for the other genotypes, *CA1* transcripts were accumulated up to 100 times more than *CA4*. Hence, *CA1* normalized transcript levels were the highest in the guard cells of Carpaccio and 6J29, but *CA4* levels were the highest in the guard cells of N38 (Figure S2h,i). The transcript accumulation of genes encoding phototropins *PHOT1* and *PHOT2* were significantly different across all genotypes. Concerning ion channels, I214 was often the genotype with the lowest level of transcripts for inward-rectifying K^+ channels (*AKT2* and *KAT3*) and proton H^+-ATPase (*OST2* and *AHA11*). Among black poplars guard cells, these

TABLE 2 Summary of the genotype, water treatment, time of day, and leaf side effect on stomatal conductance (n = 6), guard cell element content (n = 6), and expression of genes of interest (n = 4)

	Genotype (G)	Water treatment (T)	Time of day (D)	Leaf side (S)	Interaction G:T	Interaction G:D	Interaction G:S	Interaction T:D	Interaction T:S	Interaction D:S
g_s	<.001	.002	<.001	<.001	ns	.023	ns	ns	ns	ns
Na content	<.001	ns	ns	<.001	ns	ns	<.001	ns	ns	ns
Mg content	<.001	ns	.043	ns	ns	ns	ns	ns	ns	ns
P content	<.001	ns	.003	<.001	.032	ns	ns	ns	ns	ns
Cl content	<.001	.003	.009	<.001	ns	ns	<.001	ns	ns	ns
K content	<.001	ns	ns	.001	ns	ns	.015	ns	ns	ns
Ca content	<.001	ns	ns	<.001	.008	ns	ns	ns	ns	ns
An/Cat	<.001	ns	.001	.003	ns	ns	ns	ns	ns	ns
ABI1	<.001	ns	ns	.002	.004	ns	ns	ns	ns	.03
AHA11	<.001	ns	<.001	ns	.027	ns	ns	ns	ns	ns
AKT2	<.001	ns	ns	<.001	.004	.018	.001	ns	ns	ns
CA1	<.001	ns	ns	.002	.025	ns	ns	ns	ns	ns
CA4	<.001	ns	ns	<.001	ns	ns	ns	.024	ns	ns
CAX1	ns	ns	ns	<.001	ns	ns	ns	.029	ns	ns
CAX1.6	<.001	ns	ns	<.001	.042	ns	ns	ns	ns	ns
KAT1.2	<.001	ns	.031	<.001	<.001	ns	.007	ns	ns	ns
KAT3	<.001	.005	<.001	ns	ns	ns	ns	ns	ns	ns
NCED3.1	<.001	ns	.023	<.001	ns	ns	ns	ns	ns	ns
NCED3.2	<.001	ns	.049	.013	.022	ns	ns	ns	ns	ns
NHX1.13	<.001	ns	ns	ns	ns	.015	.004	ns	ns	ns
OST1	<.001	.034	.001	<.001	.01	ns	<.001	ns	ns	ns
OST2	<.001	ns	.014	ns	ns	ns	ns	ns	ns	ns
PHOT1	<.001	ns	ns	.016	ns	ns	ns	ns	ns	ns
PHOT2	<.001	ns	.011	ns	ns	ns	ns	ns	ns	ns
PIP1.2	<.001	ns	<.001	<.001	.005	ns	<.001	ns	ns	ns
PIP2.1	<.001	<0.001	.022	ns	ns	ns	ns	ns	ns	ns
PIP2.5	<.001	ns	.009	ns	ns	ns	.003	ns	ns	ns
PYL2	<.001	ns	ns	ns	ns	.048	ns	ns	ns	ns
PYL4	<.001	0.008	ns	ns	.037	ns	ns	ns	ns	ns
PYL8	<.001	ns	.032	<.001	.015	ns	.003	ns	ns	ns
SLAC1	<.001	0.018	ns	.016	.039	ns	.008	ns	ns	ns
TIP1.3	<.001	ns	.001	ns	.02	ns	ns	ns	ns	ns
TIP1.4	<.001	ns	ns	ns	.004	ns	.009	ns	ns	ns
TIP2.1	<.001	ns	.005	.049	ns	.001	ns	ns	ns	ns
TIP2.2	<.001	ns	ns	ns	ns	ns	ns	ns	ns	ns

[a]*Note*. A type two factorial analysis of variance design with first-order interactions was used. Normalized transcript levels were transformed with a logarithm function of base 2 in order to achieve a normal distribution of the data.

[b]Abbreviations: g_s, stomatal conductance to water vapour; An/Cat, ratio of anions over cations (i.e., P and Cl over Na, Mg, K, and Ca); ns, not significant. $^*P < .05$. $^{**}P < .01$. $^{***}P < .001$.

genes were either similarly or more expressed in *6J29* than in N38 (*AKT2*, *OST2*, and *SLAC1*). As to vacuolar Ca^{2+} antiporters, although transcripts accumulation for *CAX1* did not show any significant genotype effect, *CAX1.6* was on average 161 times more accumulated in the guard cells of hybrid poplars than in the black poplars. The same pattern was found for the vacuolar Na^+/H^+ antiporter *NHX1.13*. Its mRNA levels were also high in Carpaccio, compared with I214. Interestingly, *NHX1.13* expression correlated to the Na content in guard cells (Figure 4, $P < .001$, $R^2 = .35$). Targeted aquaporins (*PIPs* and *TIPs*) mRNA were consistently expressed at the lowest levels in I214, with

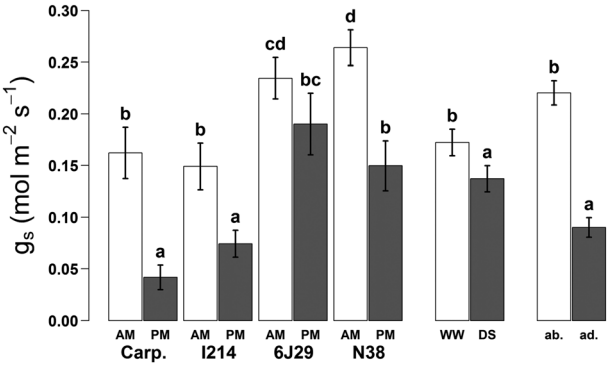

FIGURE 1 Stomatal conductance of four poplar genotypes under contrasting water availability. Each leaf side has been measured separately once in the morning and once in the afternoon (10:30 a.m. and 3:00 p.m.). Carpaccio and I214 are *Populus deltoides* × *Populus nigra*, whereas 6J29 and N38 are *P. nigra* genotypes. White and grey coloured bars show the contrasts used for statistical design. Letters show significant differences among groups for the genotype-time of day interaction, the water treatment and the leaf side ($P < .05$)

the notable exception of *TIP2.1*, which was expressed at the highest level in I214 guard cells (Figure S2u–aa).

3.3 | Impact of water availability on element content and transcript accumulation in guard cells

Cl was the only element whose content in the guard cells was significantly 13% higher in the drought-stressed trees across all genotypes (Figure S1d, $P = .003$). Moreover, the guard cells Cl content was positively correlated with g_s when controlling for genotype and water treatment differences (Figure 5, partial $R^2 = .15$, $P < .001$). Other modifications of element content in response to water regime were genotype specific. The guard cells of I214 had a P content 11% lower under drought (for well-watered and drought-stressed trees, respectively: 1.92 and 1.71 mg g^{-1}, $P = .011$, Figure S1c). Among black poplars, 6J29 and N38 exhibited an increased guard cells Ca content, respectively, 15% and 13% higher in the drought-stressed than in the well-watered trees (for control and drought-stressed, respectively, 6J29: 4.92 and 5.78 mg g^{-1}, $P = .028$; N38: 6.60 and 7.55 mg g^{-1}, $P = .015$, Figure S1f). Neither K nor any other element in guard cells were under a significant effect of the water regime, either as a main effect or as part of an interaction ($P > .075$, Table 2).

With respect to the normalized transcript accumulation, the water regime was highly genotype specific as 14 of 27 genes showed a significant interaction between the two factors (Table 2, Figure S2). Only *KAT3* and *PIP2.1* mRNA levels were significantly lower and higher, respectively, in the drought-stressed trees than in the control, in all genotypes ($P < .006$, Figure S2n–v). Concerning the hybrid poplars, the expression of three genes was down-regulated in response to water stress in the guard cells of Carpaccio (*KAT1.2*, *SLAC1*, and *TIP1.4*) but only two in the guard cells of I214 (*AHA11* and *TIP1.3*). For 6J29, the expression of *PYL8*, *CA1*, and *PIP1.2* was up-regulated in response to water stress. N38 showed the widest responsiveness to water stress. The expression of *PYL4* and *TIP1.3* was significantly down-regulated, whereas the expression of *ABI1*, *OST1*, *AKT2*, *KAT1.2*, and *PIP1.2* was up-regulated under water stress.

3.4 | Diurnal and leaf side differences of element content in guard cells

The PCA highlighted four groups consisting of every paired modality of the time of day and leaf side (Figure 6). The first and second axis explained 36.1% and 24.7% of the total variance with the third

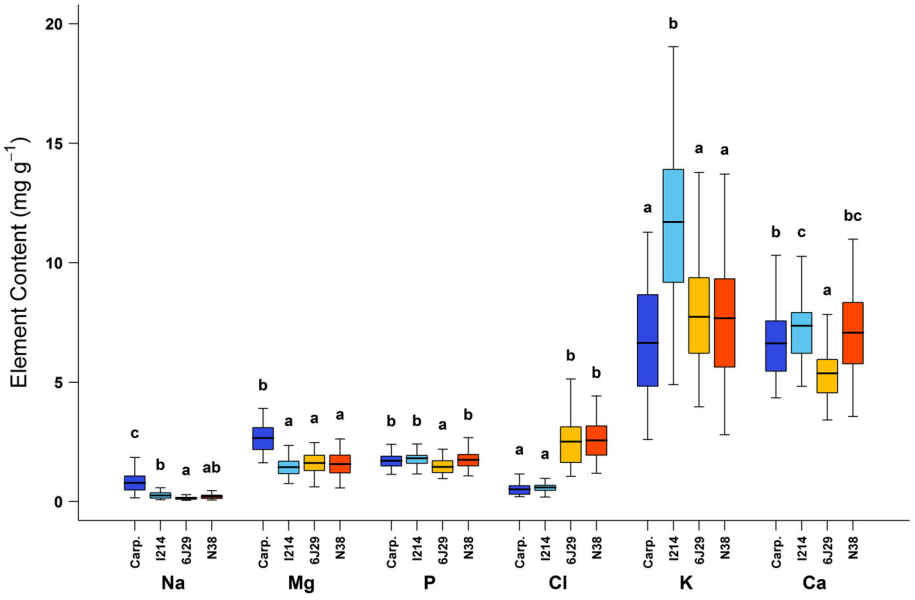

FIGURE 2 Guard cell element content of four poplar genotypes. Carpaccio and I214 are *Populus deltoides* × *Populus nigra*, whereas 6J29 and N38 are *P. nigra* genotypes (in dark and light blue, yellow and orange, respectively). Values inside each box are the genotypic mean expressed as "per mille" of dry mass, or mg g^{-1} ($n = 42–48$). Boxes and bars show the 10th, 25th, 75th, and 90th percentiles. Letters show significant differences by post hoc contrast among the four genotypes for each element [Colour figure can be viewed at wileyonlinelibrary.com]

Carp.	I214	6J29	N38	
0.00019 a	0.0001 a	0.00018 a	0.0025 b	*NCED3.1*
0.000007 a	0.0000051 a	0.0003 b	0.000002 a	*NCED3.2*
0.37 a	1 b	1.3 b	0.47 a	*ABI1*
0.13 a	0.24 b	0.61 c	0.22 b	*OST1*
0.19 a	18 d	0.93 b	8.9 c	*PYL2*
0.028 a	0.058 b	0.02 a	0.061 b	*PYL4*
0.14 a	2.3 b	0.19 a	0.26 a	*PYL8*
2.8 b	0.88 a	8 c	0.83 a	*CA1*
0.071 b	0.04 a	0.077 b	0.21 c	*CA4*
0.24 c	0.035 a	0.17 b	0.75 d	*PHOT1*
0.91 c	0.29 b	0.074 a	4.3 d	*PHOT2*
0.0036 b	0.00059 a	0.0085 b	0.00098 a	*AKT2*
0.016 a	0.019 a	0.031 b	0.034 b	*KAT1.2*
0.16 c	0.013 a	0.035 b	0.036 b	*KAT3*
0.052 d	0.0017 a	0.033 c	0.0059 b	*OST2*
0.017 b	0.0063 a	0.042 c	0.038 c	*AHA11*
0.000079 b	0.000052 ab	0.016 c	0.000015 a	*SLAC1*
0.00013 a	0.00047 a	0.0001 a	0.00017 a	*CAX1*
0.0062 b	0.0025 b	0.000033 a	0.000021 a	*CAX1.6*
0.019 d	0.0068 c	0.00039 a	0.0021 b	*NHX1.13*
0.4 d	0.023 a	0.2 c	0.071 b	*PIP1.2*
0.056 b	0.0002 a	0.46 d	0.25 c	*PIP2.1*
0.0018 b	0.0007 a	0.026 d	0.011 c	*PIP2.5*
2.2 b	0.043 a	2.7 c	4.6 d	*TIP1.3*
0.0049 b	0.0012 a	0.0021 a	0.031 c	*TIP1.4*
0.00062 ab	0.0065 c	0.0028 bc	0.00033 a	*TIP2.1*
0.000012 b	0.00000077 a	0.0000016 a	0.046 c	*TIP2.2*

FIGURE 3 Guard cell transcript accumulation heatmap of four poplar genotypes. Carpaccio and I214 are *Populus deltoides* × *Populus nigra*, whereas 6J29 and N38 are *P. nigra* genotypes. Values inside each box are back-transformed means by genotype of normalized transcript accumulation (n = 2–4). Colors range from white to orange to dark red to show the lowest, middling, and highest overall normalized expression values. Letters show significant differences by post hoc contrast among the four genotypes for each gene [Colour figure can be viewed at wileyonlinelibrary.com]

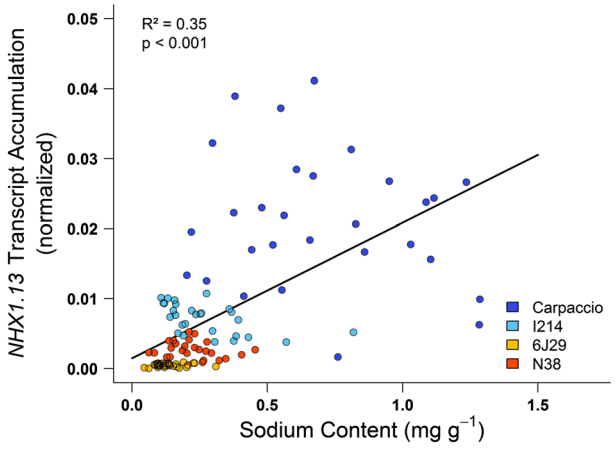

FIGURE 4 Correlation between NHX1.13 normalized transcript accumulation and sodium content (as "per mille" of dry mass) inside the guard cells of four poplar genotypes. Carpaccio and I214 are *Populus deltoides* × *Populus nigra*, whereas 6J29 and N38 are *P. nigra* genotypes (in dark and light blue, yellow and orange, respectively; n = 115) [Colour figure can be viewed at wileyonlinelibrary.com]

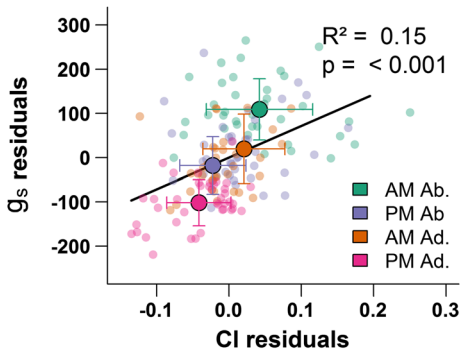

FIGURE 5 Correlation between the stomatal conductance and the chlorine content inside the guard cells once the genotype and water treatment effect is taken into account. Data used are residuals of a linear model with genotype and water treatment as main effect, chlorine content as independent variable, and stomatal conductance as dependent variable (n = 10–22) [Colour figure can be viewed at wileyonlinelibrary.com]

accounting for less than 14% (not shown). P and Ca content in the guard cells contributed the most to the first axis, whereas the *An/Cat* ratio, Cl, and K content contributed the most to the second axis. Focusing on the first two axis, the four groups formed a diagonal line parallel to the Cl and Mg content and to the supplementary variable g_s. The group associated with the abaxial side in the morning was positively related to these variables, whereas the group formed

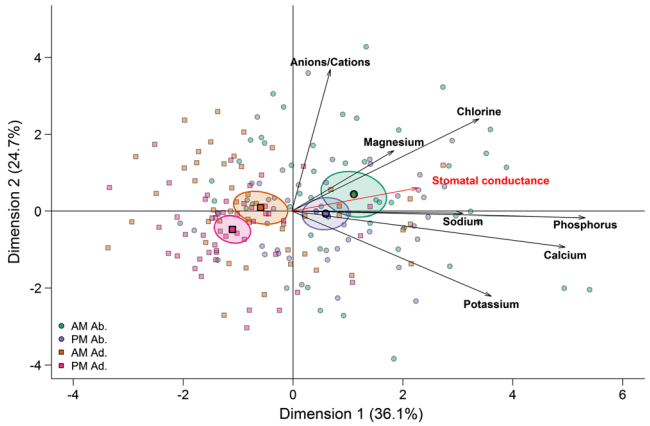

FIGURE 6 Principal component analysis of guard cell element content for each leaf side and time of day. Data used are residuals of linear models with genotype as main effect and each element content as dependent variable (n = 48). Stomatal conductance was added as a supplementary variable (in red). Mean points and confidence ellipse are shown. Circles and squares are for the abaxial and adaxial side respectively. Green and orange are for the morning and blue and pink are for the afternoon [Colour figure can be viewed at wileyonlinelibrary.com]

by the data associated to the adaxial side in the afternoon was negatively related to them. The leaf sampled in the morning showed overall a higher Mg, P, and Cl content in the guard cells and a higher *An/Cat* ratio than in the leaf sampled in the afternoon (Figure S1b,c, d,g; P = .043, P = .003, P = .009, and P < .001, respectively). The afternoon decrease ranged from 7% for P to 13% for Cl. Moreover, a higher amount of element was found in the guard cells on the abaxial side than on the adaxial side, with a noticeable exception of the Mg content (P = .07). P, Ca, and the ratio *An/Cat* were, respectively, 15%, 17%, and 10% more abundant on the abaxial side (P < .003). Cl and K were also significantly higher on the abaxial side of black poplars (Figure S1d,e). Similar trends, however not significant, were observed in hybrid poplars, as well as a 98% higher Na content in the abaxial guard cells of Carpaccio, compared with the adaxial side (Figure S1a, P < .001).

3.5 | Diurnal and leaf side differences of guard cells transcript accumulation in guard cells

Because the PCA on the guard cells element content revealed a strong structuration of sampling modalities (Figure 6), we performed a similar analysis on the gene expression for the 27 studied genes (Figure 7). The first three axis explained 21.3%, 13.1%, and 7.6% of the total variance. Among the genes contributing the most to the first axis, we found genes involved in the ABA biosynthesis and signalling pathways (*NCED3.1*, *NCED3.2*, *ABI1*, *OST1*, and *PYL8*), K^+ channels (*AKT2* and *KAT1.2*), and some of the aquaporins (*PIP1.2*, *PIP2.5*, *TIP1.4*, and *TIP2.1*). The genes contributing highly to the second axis were those encoding H^+-ATPase (*AHA11* and *OST2*), Ca^{2+} vacuolar antiporters (*CAX1* and *CAX1.6*), K^+ channels (*KAT1.2* and *KAT3*), and *NHX1.13*. Similar to the PCA on guard cells element content (Figure 6), the four modalities composed by the crossed factors leaf sides and time of day were even more clearly separated by the first two axis, compared with the element content. Morning and afternoon measures were separated on the upward diagonal, whereas leaf sides were separated on the downward diagonal. Overall, the differences between time of day were related to the expression levels of gene encoding H^+-ATPase and aquaporins, whereas the differences of leaf sides could be ascribed to genes associated mostly with the ABA response, Ca^{2+} vacuolar antiporters, and K^+ channels.

Out of the 27 genes we tested, 20 showed some degree of differential expression in the guard cells between the morning and the afternoon (Table 2, Figure S2). Only three genes were overall less expressed in the afternoon (*OST1*, *KAT1.2*, and *KAT3*) than in the morning by 16%, 17%, and 22%, respectively (Figure S2d,m,n). The remaining 17 genes were expressed at the highest level in the afternoon. This included both *NCEDs* (*NCED3.1* and *NCED3.2*), both H^+-

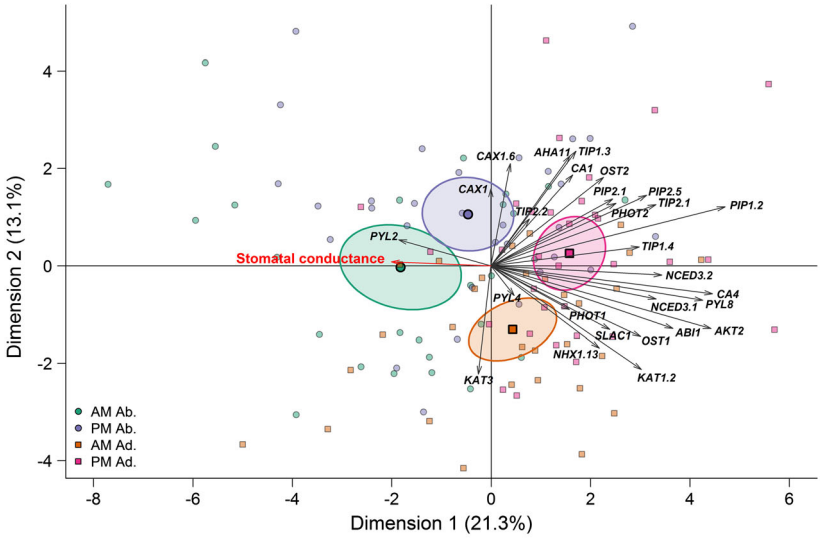

FIGURE 7 Principal component analysis of guard cell normalized transcript accumulation for each leaf side and time of day. Data used are residuals of linear models with genotype as main effect and each transcript as dependent variable (n = 30–31). Stomatal conductance was added as a supplementary variable (in red). Mean points and confidence ellipse are shown. Circles and squares are for the abaxial and adaxial side, respectively. Green and orange are for the morning and blue and pink are for the afternoon [Colour figure can be viewed at wileyonlinelibrary.com]

ATPase (*OST2* and *AHA11*), the three tested *PIPs* (*PIP1.2*, *PIP2.1*, and *PIP2.5*) as well as *PYL8*, *PHOT2*, and *TIP1.3*. *ABI1* normalized mRNA levels were higher in the afternoon but only in the guard cells on the abaxial side (Figure S2c, $P = .004$). Few significant interactions occurred between genotype and times of day. Both *NHX1.13* and *TIP2.1* transcripts levels were higher in the afternoon, but in the guard cells of hybrid poplars ($P < .05$) or of black poplars ($P < .002$), respectively (Figure S2t,z). Both *AKT2* and *PYL2* genes were more expressed in the afternoon but only in the guard cells of I214 and N38 ($P < .04$), respectively (Figure S2e,l).

With respect to the transcripts levels between leaf sides, 18 of the 27 tested genes exhibited a differential expression. In general, we observed the highest expression in the guard cells on the adaxial side of the leaf. Solely, *CAX1* and *CAX1.6* genes were, respectively, 12.6 and 10.1 times more expressed in the guard cells on the abaxial side than on the adaxial side (Figure S2r,s, $P < .001$). The same pattern was found for *TIP1.3* and for *PIP2.5* but only in I214 (Figure S2w,x). The tested *NCEDs* transcripts levels were 13.5 and 6.8 times more abundant on the adaxial side ($P < .01$). Similarly, the two ß-carbonic anhydrases (*CA1* and *CA4*), *PHOT1* and *ABI1* were also overall more expressed on the adaxial side, the accumulation of ABI1 transcripts being only significant in the morning (Figure S2c, $P = .002$). A number of genes showed a genotype-specific differential expression between leaf sides. *OST1*, *PYL8*, *AKT2*, and *KAT1.2* genes were more expressed in the adaxial guard cells only in Carpaccio and N38. *TIP1.4* mRNA level was higher in the guard cells on the adaxial side in Carpaccio. *SLAC1*, *NHX1.13*, *PIP1.2*, and *PIP2.5* genes exhibited the same pattern in N38 only. *PYL8* was also 3.3 times more expressed in the guard cells on the adaxial side than on the abaxial side of 6J29 ($P = .013$).

4 | DISCUSSION

4.1 | Genotypic diversity of element content and gene expression in the guard cells

Our study focused on the relationships between guard cell element content, expression of genes of interest, and stomatal conductance. By modulating the water regime, leaf sides, and times of day on four poplar genotypes, we intended to gain insight into the molecular drivers of gas exchange regulation and the unique physiology of guard cells.

We found a tremendous diversity for both element content and gene expression in the guard cells of four poplar genotypes. This adds to previous investigations highlighting the considerable diversity of transcript accumulation and responses to environmental factors in distinct organs of poplars (Bizet et al., 2015; Cohen et al., 2013; Dumont et al., 2014; Wilkins et al., 2009). Interestingly, Carpaccio exhibited the lowest expression of genes related to ABA biosynthesis and signalling pathways, especially compared with I214. This was not associated with a lower plasticity of stomatal closure under drought, as previous studies found a lack of stomatal response under various water deficits in I214 but not in Carpaccio (Durand et al., 2019; Muller & Lambs, 2009). Instead, exogenous supply of ABA to the I214 clone induced leaf abscission (Chen et al., 1997), which may be part of its drought response (Giovannelli et al., 2007). Additionally, we found a lower expression for the genes encoding K^+ channels, H^+-ATPase, and aquaporins in I214. These differences could in theory contribute to a reduced stomatal control by limiting the exchange of solutes and water across the plasma membrane. However, of the four genotypes studied, I214 showed the highest stomatal density and the smallest stomata (Durand et al., 2019). As such, one could expect that not only less water is required to modify the guard cell turgescence but a smaller change in aperture added over the whole leaf could result in a massive change in stomatal conductance. Durand et al. (2019) also showed that I214 had the fastest stomatal closing speed; here we found that it may not be due to an increased expression of genes encoding transporters on the guard cell plasma membrane.

Along with differences in transcript accumulation, we found evidence of a genotypic diversity of elements contributing to stomatal movements. Little is known on this topic in poplars as the majority of studies focus only on three species (Assmann, 1993), namely, *A. thaliana* (L.), *Commelina communis* (L.), and *Vicia faba* (L.). *NHX1.13* encodes a vacuolar Na^+ and K^+ transporter that has been shown to regulate cell turgor and stomatal function in *A. thaliana* (Barragan et al., 2012). The higher amount of Na in the guard cells of hybrid poplars, linked with higher *NHX1.13* mRNA levels, highlights the importance of this element for stomatal control in these genotypes. Because K^+ channels and some aquaporins may be permeable to Na^+ as well (Byrt et al., 2017; Jezek & Blatt, 2017), one could speculate that these transporters have an increased permeability for Na^+ compared with black poplars. In contrast, the higher Cl content in black poplars emphasize its role in stomatal function in these genotypes. Both malate and Cl^- act as counter-ions to balance the uptake of K^+ into the guard cells in various proportions depending on species, Cl availability, and starch metabolism (Raschke & Schnabl, 1978; Santelia & Lawson, 2016).

4.2 | Impact of water availability

A relatively weak effect of the water regime occurred on element content and normalized mRNA levels, especially as compared with the other tested factors (Table S3). This might be related to the leaf sampling, which occurred 21 days after the start of a mild drought, when short-term gene expression responses were replaced by acclimation of physiological processes. Similarly in *Populus tremula*, few changes in the expression of genes of interest were found in leaves sampled more than 20 days after the start of the drought (Possen et al., 2011). The drought response at the transcript level was highly genotype specific, in agreement with transcriptomic studies on poplar genotypes, showing divergent expression profiles under drought even when the physiological response was similar (Cohen et al., 2010; Wilkins et al., 2009). *PIP2.1* was the only up-regulated gene in all four genotypes under drought. Despite not always being the case, similar results were found on various hybrid poplars (Almeida-Rodriguez,

Cooke, Yeh, & Zwiazek, 2010; Cohen et al., 2013). *AtPIP2.1* and *ZmPIP2.1* increased water permeability when expressed in *Xenopus* oocytes and protoplasts of *Lilium longifolium* pollen (Lopez et al., 2003; Sommer et al., 2008). Moreover, *AtPIP2.1* was related to ABA-induced stomatal closure, mediated by *OST1* by increasing guard cell permeability for water enabling its efflux (Grondin et al., 2015; Maurel, Verdoucq, & Rodrigues, 2016). Increasing *PIP2.1* expression under drought may allow for a faster stomatal closure.

Under drought, there was an increased Ca content in black poplars together with a higher Cl content for all genotypes. During stomatal closure, free Ca^{2+} entering the cytosolic spaces of guard cells is known to be responsible for the inactivation of inward-rectifying K^+ channels in *V. faba* (Grabov & Blatt, 1999; Schroeder & Hagiwara, 1989) and the activation of anion channels, such as SLAC1 when expressed in *Xenopus laevis* oocytes (Geiger et al., 2010). However, Ca^{2+} elevation in the cytosol has been found to be mostly due to influx from the vacuole and the endoplasmic reticulum as opposed to entry from the apoplast (Chen et al., 2012). Unfortunately, we are not able to localize specific subcellular compartments. Concurrently to the increased Ca content, the Cl content increased as well under drought. This suggests that an increased solute concentration in guard cells may be in part a response to a decline in leaf water content in poplars under drought (Guo, Zhang, & Huang, 2010; Marron et al., 2003).

4.3 | Leaf side and time of day shape guard cell element content and transcript accumulation

Gene expression and element content in the guard cells followed opposite patterns over the leaf and along the day. Although stomatal conductance and element content reached a maximum on the abaxial side in the morning, transcript levels was overall at the lowest. The same pattern occurred, but reversed on the adaxial side in the afternoon.

There was no significant change of K content in guard cells of various apertures despite the overall element content being higher in more opened stomata, particularly with the increased Cl content (Figure 6). These results were checked by X-ray microanalyses under cryogenic conditions (−145°C). Both techniques (cryo and freeze-dried samples) showed no difference of K content inside the guard cells between leaves with markedly different stomatal conductance. The lack of decrease in K concentration in stomatal guard cells with lower stomatal aperture was quite unexpected as K is known to be of primary importance for stomatal movements. A previous study on hybrid poplar genotypes found an increased K content in guard cells under ozone while having lower g_s (Dumont et al., 2014). Langer et al. (2004) found only a 25% decrease in guard cell K content between *P. tremula* × *Populus tremuloides* leaves grown under CO_2-free environment and leaves in the dark subjected to 100-μM ABA, resulting in an 80% g_s decrease.

In our study, stomatal conductance was impacted by the different water regimes, leaf sides, and times of day. Because the difference in g_s between well-watered and drought-stressed poplars was relatively small, differences of K content would most likely also be relatively small. Consequently, such small changes may not be identified using this technique, especially when considering the many roles K has in multiple processes, from homeostasis to enzymes activation (Clarkson & Hanson, 1980). Despite a significantly lower K content on the adaxial side of the leaf, differences in g_s between leaf sides may be predominantly attributed to differences of stomatal density rather than apertures (Durand et al., 2019). Hence, differences of g_s between times of day were our best candidate to find corresponding differences in guard cell K content. However, unlike stomatal opening, it has been proposed that stomatal closure in the afternoon may be more related to sucrose than K (Amodeo, Talbott, & Zeiger, 1996; Granot & Kelly, 2019; Kottapalli et al., 2018; Talbott & Zeiger, 1996). When the rate of sucrose production is higher than its loading into the phloem, excess sucrose accumulate in the apoplast and may enter guard cells (Ewert, Jr, Zhang, Aghoram, & Riddle, 2000; Kang, Outlaw, Andersen, & Fiore, 2007), inducing stomatal closure mediated by hexokinases (Kelly et al., 2013). Clearly, more research is needed to decipher changes of element content in poplar guard cells related to stomatal movements, which will necessitate the input of multiple techniques.

Diurnal rhythms in transcript accumulation were reported by previous studies for the expression of aquaporin-encoding genes and genes involved in ABA regulation. In contrast to our findings, whole leaves and roots generally have an increased accumulation of aquaporin transcripts in the morning, or in the few hours following an increase of light (Hachez, Heinen, Draye, & Chaumont, 2008; Henzler et al., 1999; Lopez et al., 2003; Lopez et al., 2013; Vandeleur et al., 2009). On the other hand, even if leaf ABA concentrations tend to increase at the end of the day (Novakova et al., 2005), the genes involved in the ABA-related pathways seem to present a diversity of diurnal rhythms in leaves (Seung et al., 2012). Despite their close proximity to mesophyll cells, stomata have specific circadian rhythms (Hassidim et al., 2017; Yakir et al., 2011). Given their relatively low proportion in the leaf compared with other cell types, the different expression profile along the day in guard cells compared with whole leaves could result from the cellular complexity of the organ that hides cell-type specific regulations (Nourbakhsh-Rey & Libault, 2016). In addition to the changes in element content, guard cells are also subjected to pH variations during stomatal movement. In general, stomatal closure induces cytosolic and vacuolar alkalinization (Irving, Gehring, & Parish, 1992; Zhang, Dong, Gao, & Song, 2001), whereas stomatal opening induces acidification (Huang, She, Zhang, & Zhao, 2013), linked with H_2O_2 and Ca^{2+} signalling during both ABA and dark-induced stomatal responses (Agurla & Raghavendra, 2016; Ma & Niu, 2017). Moreover, external pH changes strongly altered transcript levels, which was linked to ABA-responsive element motifs in the promoter region of pH-responsive genes (Lager et al., 2010). Little is known about the effect of intracellular pH on transcript accumulation, and whether or not guard cell movements affect its own expression pattern remains to be established.

Potential differences in the physiology of guard cells between leaf sides is seldom investigated, and even fewer studies compare the

patterns of transcript accumulation and/or element content. We reported here an overall stronger expression of genes of interest on the adaxial side for a number of genes, including *NCEDs* and ß-carbonic anhydrases. However, the higher expression of vacuolar Ca^{2+} antiporters on the abaxial side was associated with a higher Ca content on this side, in agreement with Dumont et al. (2014). It has been hypothesized that guard cells on each side may have different pathways for Ca^{2+}-mediated signal transduction (Wang, Wu, & Assmann, 1998). There is evidence of an independent control of gas exchange between leaf sides (Mott, Cardon, & Berry, 1993; Richardson et al., 2017). Such control would allow amphistomatal leaves to regulate more efficiently in response to the environmental conditions specific to each side. Previously reported thermal and irradiance gradients between leaf surfaces of a few degrees (Buckley, John, Scoffoni, & Sack, 2015; Clum, 1926; Sheriff, 1979) could result in different perceived environmental conditions between leaf sides (e.g., light, VPD, and temperature), prompting different stomatal control, as was found on poplar clones in response to a change in light and VPD (Ceulemans, Hinckley, & Impens, 1989; Pallardy & Kozlowski, 1979). Differences in the intensity and nature of perceived stresses, such as evaporative demand, light, and heat (Urban, Ingwers, McGuire, & Teskey, 2017) may induce a distinct conjugation of stress between sides, which may affect the synergic or antagonistic response to environmental cues (Bigot et al., 2018). This would result in a different perception and signalization between sides; for example, stomata of *Gossypium barbadense* (L.) have distinct sensitivity to light between sides linked with different pigment contents (Lu, Quiñones, & Zeiger, 1993). A different sensitivity between sides would lead to a diversity of gene expression (see Bigot et al., 2018), and element content in the guard cells, which may be a part of what we observed. A stronger gene expression on the adaxial side might be seen as the result of a more extreme condition on the side facing the light source.

In conclusion, even though we found a strong genotypic diversity, our data show how changes in stomatal conductance between leaf sides and time of day are linked to guard cell element content and transcript accumulation profiles in a similar way across the four studied genotypes (Figures 6 and 7). Future technical developments such as cryogenic electron microscopy will allow for an easier element cellular compartmentation. Because stomata are responsible for optimizing carbon gain with regard to water loss, their functions affect not only the plant but the ecosystem as well (Berry, Beerling, & Franks, 2010). Understanding the specificities of guard cells functioning will further expand our current knowledge of stomatal physiology and their global consequences.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTION

D. L. T., O. B., C. B., M. D., and D. C. developed the experimental design. C. B. carried out the experimental drought set-up. D. L. T. collected leaf porometer data; D. L. T., D. C., I. H., and N. A. sampled the leaves; M. D. performed X-ray microanalysis; M. D., D. C., and I. T. microdissected the stomata and extracted the RNA. N. A. performed cDNA amplification, RT-PCR, and data standardization. M. D., D. L. T., D. C., and I. H. performed data analysis. All contributors were involved in the writing and reviewing of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 List of gene studied with their specific primers.

Table S2 Dataset used for statistical analysis (see separate Excel file).

Table S3 Summary of the genotype, water treatment, time of day and leaf side partial R^2 of stomatal conductance, guard cell element content and expression of genes of interest.

Figure S1 Guard cell element content of four poplar genotypes under contrasting water availability with each leaf side measured separately once in the morning and once in the afternoon (10:30 and 15:00).

Figure S2 Normalized guard cell transcript accumulation of four poplar genotypes under contrasting water availability with each leaf side measured separately once in the morning and once in the afternoon (10:30 and 15:00).

Data S2: Supplementary Information

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