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The plasma membrane aquaporin ZmPIP2;5 enhances the sensitivity of stomatal closure to water deficit

Lei Ding[1] | Thomas Milhiet[1] | Boris Parent[2] | Adel Meziane[2] | François Tardieu[2] | François Chaumont[1]

[1]Louvain Institute of Biomolecular Science and Technology, UCLouvain, Louvain-la-Neuve, Belgium

[2]INRAE, LEPSE, Université de Montpellier, Montpellier, France

Correspondence
François Chaumont, Louvain Institute of Biomolecular Science and Technology, UCLouvain, 1348 Louvain-la-Neuve, Belgium.
Email: francois.chaumont@uclouvain.be

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Abstract

Increasing stomatal movement is beneficial to improve plant water use efficiency and drought resilience. Contradictory results indicate that aquaporins might regulate stomatal movement. Here, we tested whether the maize plasma membrane PIP2;5 aquaporin affects stomatal closure under water deficit, abscisic acid (ABA) or vapour pressure deficit (VPD) treatment in intact plants, detached leaves or peeled epidermis. Transpiration, stomatal conductance (g_s) and aperture and reactive oxygen species (ROS) in stomatal complexes were studied in maize lines with increased or knocked down (KD) *PIP2;5* gene expression. In well-watered conditions, the PIP2;5 overexpressing (OE) plants transpired more than wild types (WTs), while no significant difference in transpiration was observed between *pip2;5* KD and WT. Upon mild water deficit or low ABA concentration treatments, transpiration and g_s decreased more in PIP2;5 OE lines and less in *pip2;5* KD lines, in comparison with WTs. In the detached epidermis, ABA treatment induced faster stomatal closing in PIP2;5 OE lines compared to WTs, while *pip2;5* KD stomata were ABA insensitive. These phenotypes were associated with guard cell ROS accumulation. Additionally, PIP2;5 is involved in the transpiration decrease observed under high VPD. These data indicate that maize PIP2;5 is a key actor increasing the sensitivity of stomatal closure to water deficit.

KEYWORDS
abscisic acid, facilitated membrane water diffusion, reactive oxygen species, stomatal conductance, transpiration, vapour pressure deficit

1 | INTRODUCTION

The kinetics and magnitude of stomatal aperture/conductance are affected by natural environmental fluctuations, such as light, soil water availability, CO_2 and air vapour pressure deficit (VPD) (Sussmilch et al., 2019; Tardieu & Simonneau, 1998; Zhang et al., 2018). A faster stomatal movement in response to the changing environment was shown to improve water use efficiency and plant growth without penalty in carbon fixation (Lawson & Blatt, 2014; Papanatsiou et al., 2019). In dry environments, including dry soil and atmosphere, stomatal closure is mediated by abscisic acid (ABA) signalling in guard cells (Cai et al., 2017; Pantin & Blatt, 2018; Sussmilch et al., 2019) and bundle sheath cells (Moshelion et al., 2015; Pantin et al., 2013). In the latter cells, ABA signalling triggers a decrease in leaf hydraulic conductance (K_{leaf}) and leaf water potential (Ψ_{leaf}), contributing to stomatal closure via a hydraulic mechanism (Pantin et al., 2013). In stomata, ABA signalling activates the membrane transport system, including potassium and anion channels, and possibly aquaporins, to decrease guard cell turgor pressure and volume, leading to stomatal closure (Grondin et al., 2015; Jezek & Blatt, 2017; Papanatsiou et al., 2019).

For many years, the roles of aquaporins in controlling stomatal movement remained hypothetical, even if their role in facilitating membrane diffusion of water and small solutes makes them putative actors for guard cell turgor pressure adjustment (Chen et al., 2017; Ding & Chaumont, 2020; Hachez et al., 2017; Jezek & Blatt, 2017). The first direct evidence highlighted the involvement of *Arabidopsis* AtPIP2;1 in ABA-induced stomatal closure, as the closure was impaired in at*pip2;1* knockout (KO) lines (Grondin et al., 2015). In addition, the ABA-activated OST1 kinase phosphorylates the Ser-121 of AtPIP2;1, an event known to activate PIP aquaporin water channel activity (Grondin et al., 2015). AtPIP2;1 also facilitates H_2O_2 diffusion into guard cells to mediate ABA- and pathogen-triggered stomatal closure (Rodrigues et al., 2017). At the same time, AtPIP2;1 was suggested to be involved in stomatal closure induced by higher CO_2 concentrations (C. Wang et al., 2016). Phosphorylated AtPIP2;1 was also shown to transport cations when expressed in heterologous expression systems (Qiu et al., 2020). Altogether, these results indicate that AtPIP2;1 has multiple substrate specificities (water, H_2O_2, CO_2 and potentially cations including K^+) that might regulate stomatal movement (Ding & Chaumont, 2020). However, the ABA-induced stomatal closure phenotype of the *Arabidopsis atpip2;1* KO line could not be reproduced, and even a quadruple *atpip1;1*, *atpip1;2*, *atpip2;1* and *atpip2;2* mutant did not exhibit any difference in stomatal closure and conductance (Ceciliato et al., 2019; C. Wang et al., 2016). Further evidence is therefore required to ascertain the exact role(s) of aquaporins in stomatal movement in plants (Ding & Chaumont, 2020).

In this study, we examined whether the deregulation of maize *PIP2;5* gene expression affects the time course of stomatal closure induced by water deficit, ABA treatments or high VPD in intact plants, detached leaves and peeled epidermis. We previously showed that PIP2;5 is the main actor in controlling cell and tissue hydraulic conductivity, as well as in controlling plant growth under water deficit conditions (Ding et al., 2020; Hachez et al., 2012). Additionally, H_2O_2 membrane diffusion is enhanced by expressing PIP2;5 in yeast (Bienert et al., 2014). Here, we report that PIP2;5 affects early stomatal closure and transpiration decrease under water deficit or high VPD.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

Two PIP2;5 overexpressing (OE) and one *pip2;5* knocked down (KD) maize lines used in this study were described in Ding et al. (2020). While the decrease in *PIP2;5* expression was dramatic in the *pip2;5* KD line, a very low expression was still detected (Ding et al., 2020). The heterozygous plants were self-pollinated and, in the next generation, the homozygous plants were identified by qRT-PCR (PIP2;5 OE lines), and by PCR on genomic DNA (*pip2;5* KD lines). Homozygous plants segregating for the *PIP2;5* transgene (PIP2;5 OE) and nontransgenic siblings (WT-B104) or *pip2;5* KD and siblings without the Mu transposon (WT-W22) were utilized. Plants were grown in soil in a growth chamber or in a greenhouse (experiments detailed in Table 1). The growth chamber was with a 16 h/8 h light/dark cycle (25/18°C) and a daytime light intensity of 200 µmol m^{-2} s^{-1} at the leaf top. The chamber humidity during the light cycle was 50%–70%.

2.2 | In situ immunolocalization and western blot

The epidermis was peeled from leaves of 2-week-old maize plants (PIP2;5 OE-4, PIP2;5 OE-13, *pip*2;5 KD and their corresponding wild type [WT] plants). In situ immunolocalization of PIP2;5 and plasma membrane H^+-ATPases (PMA) was performed as previously described (Hachez et al., 2006). The fluorescence signal was detected by confocal microscopy (LSM 710; Carl Zeiss) using a C-Apochromat ×40/1.20 water immersion objective with an excitation at 488 nm and an emission at 510–530 nm. PIP2;5 preimmune serum was used

TABLE 1 Experiments carried out in this study

Experiments	Lines	Place	Treatments	Measured parameters
1	PIP2;5 OE-4, WT-B104	Growth chamber, LLN	Well watered to water deficit (stop watering)	Tr
2	PIP2;5 OE-4(13), WT-B104, *pip2;5* KD, WT-W22	Growth chamber, LLN	Well watered, mild water deficit (ψ_{soil} = −0.1 to −0.2 MPa)	Tr
3	PIP2;5 OE-4(13), WT-B104	Growth chamber, LLN	Severe water deficit (ψ_{soil} = −0.3 to −0.5 MPa)	Tr
4	PIP2;5 OE-4(13), WT-B104, *pip2;5* KD, WT-W22	Growth chamber, LLN	ABA treatments with detached leaves or peeled epidermis	g_s, stomatal aperture, ROS in guard cells
5	PIP2;5 OE-4, WT-B104	Growth chamber, Montpellier	Well watered, severe water deficit (ψ_{soil} = −0.45 to −0.6 MPa), low VPD (~1 kPa), high VPD (~2.5 kPa)	Tr

Abbreviations: ABA, abscisic acid; g_s, stomatal conductance; LLN, Louvain-la-Neuve; ROS, reactive oxygen species; Tr, transpiration; VPD, vapour pressure deficit.

as a negative control for the first antibody. Western blots were performed with protein extracts from peeled epidermis as described in Ding et al. (2020). The fluorescent signal intensity in stomatal complexes and PIP2;5 signals from Western blot were quantified with Image J software. The PIP2;5 signals from the Western blot were normalized with the PMA levels.

2.3 | Whole plant transpiration

Whole plant transpiration was monitored continuously for two PIP2;5 OE (PIP2;5 OE-4 and PIP2;5 OE-13) and one *pip2;5* KD lines, together with the respective WT siblings (WT-B104 and WT-W22) (Ding et al., 2020), under well-watered conditions and soil water deficit. It was determined by gravimetry in a growth chamber. Briefly, 3-week-old maize plants were placed on a balance (FX-3000i; A&D) and the weight was recorded every 10 min with a weighing datalogger (AD-1688; A&D) in the light/dark cycle. To avoid soil evaporation, the pots were covered with aluminium foil. After measurement, the leaf was scanned and the leaf area was analysed with ImageJ software. Whole plant transpiration was calculated with the weight loss and normalized with the leaf area. Soil water potential was measured by WP4C DewPoint Potentiometer (Decagon Devices Inc.). For the measurements under progressively drying soil conditions, the pots were firstly watered at saturation. Then, the pot weight was recorded continuously after stopping watering. Soil water potential was measured and the calibration was performed by plotting pot weight versus water potential. For mild and severe water deficit conditions, we stopped watering the pots for 3–4 days and adjusted the water potential to −0.1 to −0.2 MPa and −0.3 to 0.5 MPa, respectively, by adding water 3 h before the transpiration measurements.

The transpiration under different VPD and soil water potential was conducted in the high-throughput phenotyping platform Phenodyn of the LEPSE laboratory in Montpellier (Sadok et al., 2007). The plants were grown in a growth chamber as described in Ding et al. (2020). During the measurements, two VPDs were applied during the day. At the beginning of the day (7:00 AM in solar time), VPD was set at 1 kPa for 4 h, changed to 3 kPa for 6 h (from 11:00 AM to 17:00 PM) and then at 1 kPa for 4 h of VPD (until 21:00 PM). During the night (from 21:00 PM to 7:00 AM), the VPD was 0.5 kPa. Temperatures were set at 28°C during the day and 20°C during the night.

2.4 | Stomatal conductance (g_s)

g_s was measured with a Li-Cor 6400XT portable photosynthesis system (Li-Cor Inc.). In g_s measurement under ABA treatments, the third leaf from 2-week-old maize plants growing in growth chamber was excised with ~3 cm leaf sheath, which was recut inside Milli-Q water to have a final leaf with ~2 cm leaf sheath. Then, the excised leaf was transferred to a 5-ml Eppendorf tube filled with milli-Q water. This transfer was performed in water to avoid any air entering into the leaf. The leaf was stabilized under light for ~30 min and then placed in the Li-Cor 6400XT system leaf chamber. During the measurement, the chamber temperature was maintained at ~28°C and the light intensity was 1500 µmol m^{-2} s^{-1} (with 10% blue + 90% red light). CO_2 concentration was set at 400 µmol mol^{-1} controlled with a CO_2 cartridge (Liss). The relative humidity was 40%–50%. g_s was recorded every 30 s for ~20 min before ABA was added, and then for another ~30 min with the indicated ABA concentrations.

2.5 | Stomatal aperture

Stomatal aperture was measured from fourth leaf abaxial epidermal peels from 3-week-old maize plants growing in a growth chamber. First, the fully expanded leaf was excised and cut into ~2-cm-long sections, with the abaxial side floated on the incubation buffer (10 mM KCl, 50 µM $CaCl_2$, 10 mM MES, pH 5.6 (Tris)) (Gao et al., 2017) (Greiner Bio-One). The leaves were exposed to light (~200 µmol m^{-2} s^{-1}) for 2 h to induce stomatal opening. Then, the leaf epidermis was peeled and the strips were floated on the incubation buffer containing either 10 µM ABA (Sigma-Aldrich) (30 mM ABA stock in ethanol) or ethanol as a control (0.03% (v/v)). Stomatal aperture was checked after 30, 60 and 120 min treatments with a fluorescence microscope (Axio observer 7; Zeiss) equipped with a CCD camera (ORCA-Flash 4.0 LT C11440; Hamamatsu). The aperture size was measured with ImageJ software.

2.6 | ROS in stomata

Reactive oxygen species (ROS) in stomata was detected by H_2DCFDA staining (Iwai et al., 2019; Pei et al., 2000). Stomatal opening was induced as above. After 2 h of induction, the epidermis was peeled inside the incubation buffer, and the strip was submerged into the incubation buffer with either 10 µM ABA or 0.03% (v/v) ethanol used as control. After the indicated time, the epidermal strip was washed three times with Milli-Q water and stained with 50 µM H_2DCFDA (Sigma-Aldrich) (10 mM stock in dimethyl sulfoxide) in incubation buffer for 20 min in the dark. The strip was washed three times and the fluorescence signal was detected by microscopy (excitation: 494 nm; emission: 517 nm) and quantified with ImageJ software.

2.7 | Statistical analyses

Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software). Student's *t* test was applied to determine the significant differences in Figures 1, 3, 4 and 5. One-way analysis of variance (ANOVA) with Tukey's posttest was applied to compare the significant differences in Figures 6 and 7.

3 | RESULTS

3.1 | *PIP2;5* expression in stomatal complexes of *PIP2;5* OE and KD lines

PIP2;5 expression was detected in whole tissues of maize leaf mature zone, epidermis and stomatal complexes, but at lower levels compared with its expression in root tissues (Hachez et al., 2006, 2008; Ding et al., 2020; Heinen et al., 2014). In situ immunolocalization showed here that PIP2;5 abundance was high in epidermal and stomatal subsidiary cells in both OE lines (PIP2;5 OE-4, Figure 1c,d; PIP2;5 OE-13, Figure 1e,f), while it was low in WT-B104 (Figure 1a,b). A twofold higher signal was observed in both PIP2;5 OE lines than WT-B104 (Figure 1m). Two layers of fluorescence signal were observed, indicating that the PIP2;5 localized in both subsidiary and epidermal cells (Figure 1a,c,e,g,i, inset). In contrast, a low signal was observed in the guard cells of PIP2;5 OE lines. The PIP2;5 signal in *pip*2;5 KD stomatal complexes was twofold lower than in WT-W22 (Figure 1g–j,n). The absence of PIP2;5 signal in OE-4 epidermal cell membranes when using the preimmune serum (Figure 1k,l), indicated that the signals obtained using the anti-PIP2;5 serum were specific for PIP2;5, as previously reported (Hachez et al., 2006).

A high abundance of PIP2;5 proteins in the two studied PIP2;5 OE lines was observed by Western blot analysis, while no signal was detected in the WT-B104, WT-W22 and *pip*2;5 KD samples (Figure 1o). However, when the latter extracts were loaded separately, a longer exposure time of the membrane allowed detection of PIP2;5 in WT-B104, WT-W22 and *pip2;5* KD samples (Figure 1p). The relative PIP2;5 signal in *pip*2;5 KD epidermis decreased by 36% when compared with the WT-W22 (Figure 1q).

3.2 | *PIP2;5* gene expression deregulation affects the transpiration rate

We first measured transpiration with PIP2;5 OE-4 and WT siblings (WT-B104) grown in progressively drying soil conditions after stopping watering (Figure 2). Daytime transpiration was first higher in

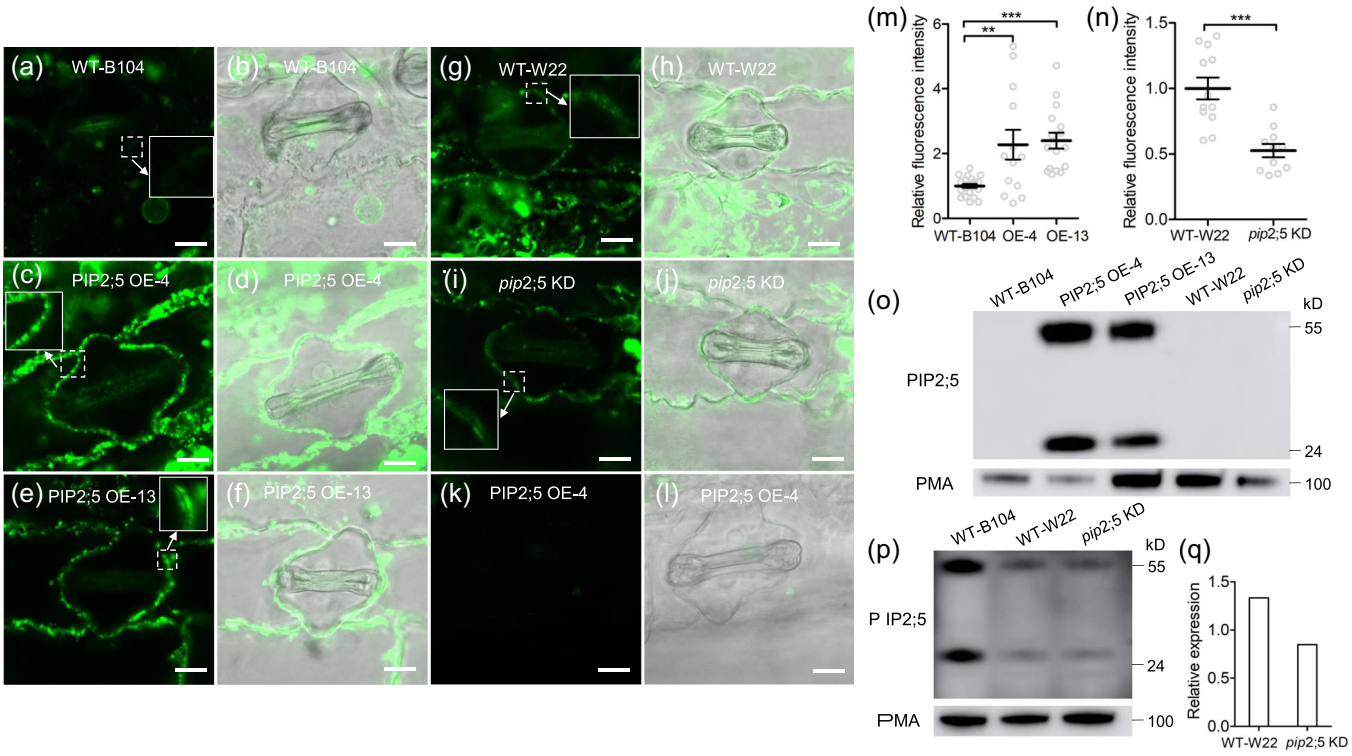

FIGURE 1 PIP2;5 abundance in the epidermis and stomatal complexes of PIP2;5 deregulated maize lines. (a–n) PIP2;5 immunolocalization in epidermal strip of WT-B104 (a,b), PIP2;5 OE-4 (c,d,k,l), PIP2;5 OE-13 (e,f), WT-W22 (g,h) and *pip*2;5 KD (i,j) plants. The PIP2;5 preimmune serum was used as a negative control in (k and l). Scale bars = 10 µm. (m, n) Quantification of the PIP2;5 fluorescence signals in the stomatal complexes of PIP2;5 OE (m) and *pip*2;5 KD lines (n). The results were the relative signal intensity compared to the WT plants from two independent experiments and 11–22 stomatal complexes were used for the quantification. Data are means ± standard error. Student's *t* test was applied to compare the significant difference of PIP2;5 signals between WT and PIP2;5 OE or *pip*2;5 KD plants at the level of $^{**}p < 0.01$ and $^{***}p < 0.001$. (o–q) PIP2;5 protein levels in the PIP2;5 deregulated maize epidermis. (q) Quantification of the PIP2;5 signal from the Western blot of WT-W22 and *pip*2;5 KD samples, relative to the PMA signal. The epidermis was peeled from two to four maize plants (2-week-old) and total protein was extracted. Five micrograms (o) and 10 µg (p) of total proteins were loaded on the gel. Western blots were performed with antibodies raised against PIP2;5 and PMA. Two PIP2;5 bands corresponding to the monomeric (low) and dimeric (upper) forms were detected. KD, knocked down; OE, overexpressing; PMA, plasma membrane H^+-ATPases; WT, wild type [Color figure can be viewed at wileyonlinelibrary.com]

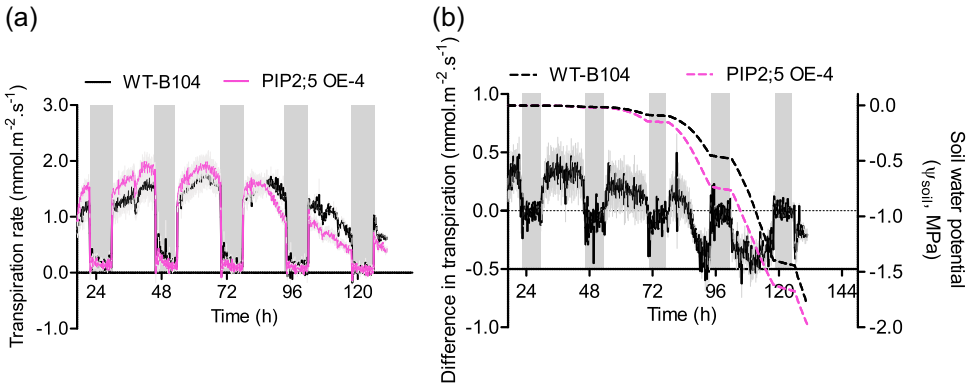

FIGURE 2 Transpiration of maize lines overexpressing PIP2;5 growing in a growth chamber (experiment 1, Table 1). (a) Transpiration of 3-week-old WT-B104 and PIP2;5 OE-4 plants was recorded continuously after withholding irrigation for 5 days. (b) Transpiration difference between PIP2;5 OE-4 and WT-B104 plants. The soil water potential is indicated by the dashed lines. The mean transpiration rate was calculated from three plants and the error bar indicated the SE. Grey background represents the dark periods. OE, overexpressing; WT, wild type [Color figure can be viewed at wileyonlinelibrary.com]

PIP2;5 OE-4 plants than in WT when soil water potential was still close to 0 (well-watered plants). Because soil water was depleted more rapidly by the rapidly transpiring OE than by WT plants (Figure 2b), transpiration rate became lower in OE plants than in WT after 80 h (Figure 2a).

Daily time courses presented in Figure 3 showed a similar trend. In this experiment, we compared the transpiration of all *PIP2;5* deregulated plants for one day with similar starting soil water potential, either close to zero (well-watered) or −0.1 to −0.2 MPa (mild water deficit) (see 'Materials and methods'). In well-watered conditions, PIP2;5 OE-4 and OE-13 plants transpired 22% and 14% more, respectively, than WT-B104 (Figure 3a,c), whereas *pip2;5* KD and WT-W22 plants had similar daytime transpiration (Figure 3e). The opposite trend was observed in mild water deficit, in which PIP2;5 OE-4 and OE-13 plants transpired 16% and 9% less than WT-B104 (Figure 3b,d and Figure S1a), whereas *pip2;5* KD plants transpired 31% more than WT-W22 (Figure 3f). The observation that transpiration of PIP2;5 OE was still higher than WT at −0.1/−0.2 MPa in progressive soil drying experiment (Figure 2) and not when this soil water potential was imposed (Figure 3) suggest that, in the latter case, PIP2;5 OE plants adapted to mild water deficit.

Under severe soil water deficit conditions ($\Psi_{soil} \approx -0.3$ to −0.5 MPa), PIP2;5 OE plants transpired more than the WT-B104 plants (Figure S1), indicating that the OE plants react differently depending on the soil water deficit conditions (Ding et al., 2020).

To determine whether PIP2;5 overexpression modified plant transpiration in response to high evaporative demands, that is, high VPD, we measured WT-B104 and PIP2;5 OE-4 transpiration rates in an experiment where VPD ranged during the day between 1 kPa in the morning and late afternoon to 2.2 kPa in the middle of the day, thereby mimicking changes in VPD during the day in natural conditions. This was performed in well-watered conditions and in the severe water deficit ($\Psi_{soil} \approx -0.4$ to −0.6 MPa) that caused higher transpiration in OE plants compared with WT (Figure 4). For both lines, the transpiration rate was higher under high VPD (~2.5 kPa) than low VPD (~1 kPa) independently of soil water potential (Figure 4). In well-watered conditions, the transpiration rate of PIP2;5 OE plants was first higher in low VPD (first hours after light onset) than in WT-B104 plants in accordance with the previous results. This order was reversed under high evaporative demand, with higher transpiration rates in WT-B104 than in PIP2;5 OE plants. The difference in transpiration rates between OE and WT plants in severe water deficit, observed in the first experiment (Figure S1), was increased under high evaporative demand (Figure 4b). PIP2;5 OE transpiration tends to decrease with continuous high VPD treatment under both well water and severe water deficit conditions, while the transpiration of WT plants was stable.

Altogether, these data suggest that PIP2;5 OE plants have higher transpiration rates in both well-watered and severe water deficit, while at mild water deficit and high VPD conditions, they decreased their transpiration rate compared with the WT plants.

3.3 | ABA-induced stomatal closure is altered by *PIP2;5* gene expression deregulation

In excised leaves, 0.1 µM ABA application in the incubation buffer caused a faster decrease in stomatal conductance (g_s) in the two OE lines than in WT, whereas the opposite was observed in the KD line (Figure 5). However, this difference was not observed when using 0.5 µM ABA (Figure S2). The stomatal aperture (pore width; Figure S3) was also measured in the peeled epidermis in the absence or presence of ABA for the different maize lines (Figure 6). A significant decrease in stomatal aperture was measured in the peeled epidermis of both PIP2;5 OE lines after 30 min incubation with 10 µM ABA (Figure 6a,b), while this was not observed in WTs, which showed a significant decrease after 60 min only (Figure 6a,b). In contrast, the ABA treatments even slightly increased the stomatal aperture of *pip*2;5 KD line compared with the control treatment (Figure 6c).

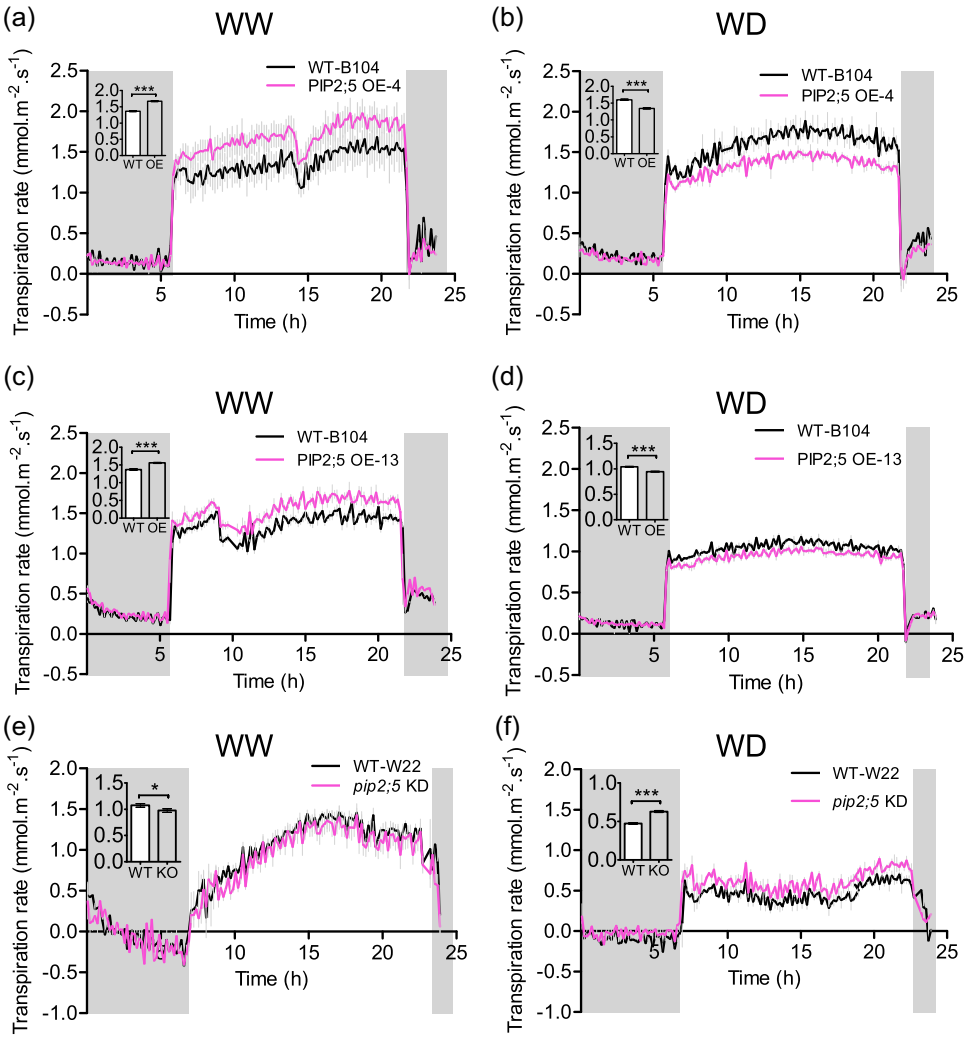

FIGURE 3 Transpiration during one light–dark cycle under well-watered (WW) conditions (a,c,e) and mild soil water deficit (WD) (b, d, f) of plant growing in a growth chamber (experiment 2, Table 1). (a) and (b) PIP2;5 OE-4 lines; (c) and (d) PIP2;5 OE-13 lines; (e) and (f) *pip2;5* KD lines. The insets indicate the day mean transpiration rate. The transpiration rate was calculated at each 10 min time point, and two to five plants were measured for each genotype. All the values coming from the different time points were analysed together to obtain the day mean transpiration rate for all the plants with the same genotype. The dark period is shown by the grey bar. The soil water potential was ~−0.10 MPa in mild soil water deficit treatment. The mean values were from two to five plants (Figure 1a,b, two WT-B104 plants and three PIP2;5 OE-4 plants; Figure 1c, four WT-B104 plants and three PIP2;5 OE-13 plants; Figure 1d, two WT-B104 plants and five PIP2;5 OE-13 plants; Figure 1e, four WT-W22 plants and four *pip*2;5 KD plants; Figure 1f, three WT-W22 plants and three *pip*2;5 KD plants). The error bars indicate the SE. Student's *t* test was applied to compare the significant difference of day mean transpiration between the lines at levels of ***$p < 0.001$ and *$p < 0.05$. KD, knocked down; ns, not significant; OE, overexpressing; WT, wild type [Color figure can be viewed at wileyonlinelibrary.com]

3.4 | ROS accumulation induced by ABA is altered by PIP2;5 deregulation

ABA-induced stomatal closure is dependent on ROS (mainly H_2O_2) accumulation in guard cells (Pei et al., 2000; Rodrigues et al., 2017). We followed H_2O_2 accumulation in guard cells after epidermis incubation with ABA using the H_2DCFDA probe. Significant H_2O_2 accumulation was measured in PIP2;5 OE guard cells after 2 min ABA (10 μM) incubation, while no accumulation was detected in WT-B104 after this short time (Figure 7a,b,d,e). A significant increase in the H_2O_2 signal in WT guard cells was only found after 15 min ABA treatment. On the other hand, no ROS accumulation was observed in *pip2;5* KD guard cells at any ABA incubation time, while the signal increased after 15 min ABA treatment in the WT-W22 siblings (Figure 7c,f).

4 | DISCUSSION

4.1 | PIP2;5 affects transpiration in a water condition-dependent manner

Water flow and stomatal aperture are regulated by aquaporins expressed in roots and shoots, which, in turn, would impact the overall plant transpiration. Transpiration and/or g_s changes up to 40%

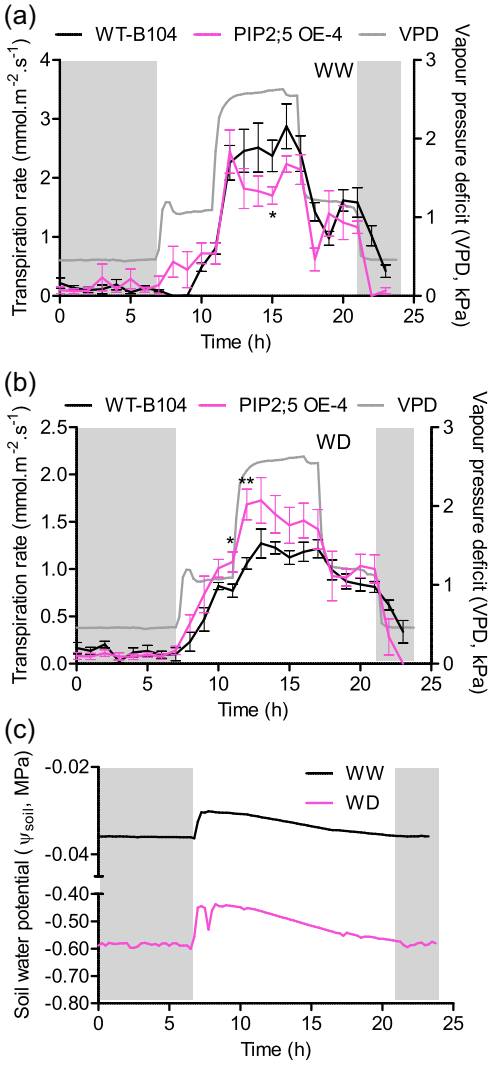

FIGURE 4 Transpiration under different VPD and soil water potential of plants growing in a growth chamber (experiment 5, Table 1). (a) Transpiration of PIP2;5 OE-4 and WT plants under well-watered (WW) conditions. (b) Transpiration of PIP2;5 OE-4 and WT plants under water deficit (WD) conditions. (c) Soil water potential of WW and WD treatments. Grey lines in (a) and (b) indicate the time course of VPD in the growth chamber. The mean values were from three plants. The error bars indicate the SE. Student's *t* test was applied to compare the significant difference in transpiration rate between PIP2;5 OE-4 line and WT at the levels of $^{*}p < 0.05$ and $^{**}p < 0.01$. OE, overexpressing; VPD, vapour pressure deficit; WT, wild type [Color figure can be viewed at wileyonlinelibrary.com]

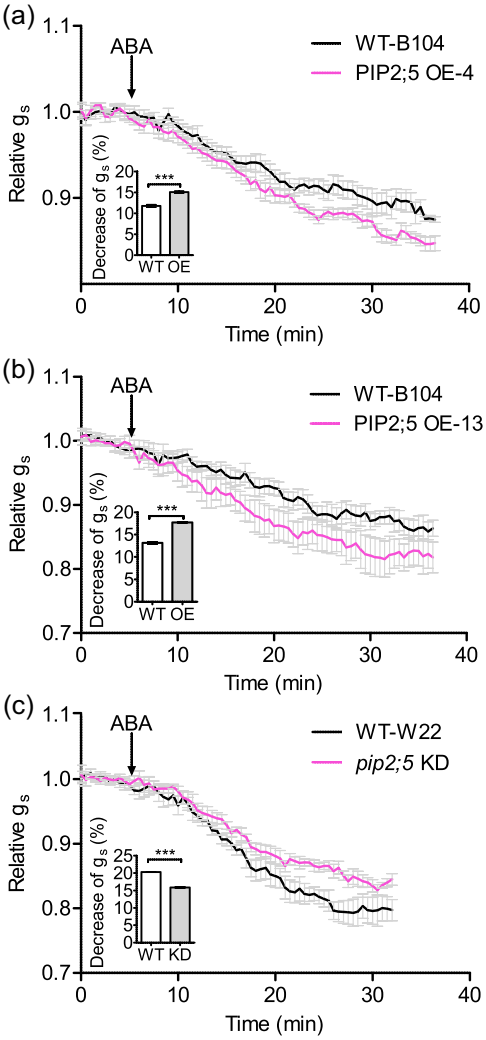

FIGURE 5 Time course of stomatal conductance (g_s) after an ABA treatment of plants growing in a growth chamber (experiment 4, Table 1). (a) PIP2;5 OE-4, (b) PIP2;5 OE-13 and (c) *pip2;5* KD. The black arrows indicate the time of 0.1 μM ABA addition. The mean g_s values were calculated 5 min before ABA addition, and the data were normalized with these mean values. The data were calculated from 5 to 9 leaves from two independent experiments. The insets indicate the g_s percentage decrease, calculated from the measurement of the last 5 min. Student's *t* test was applied to compare the significant difference in g_s decrease between the lines at the $^{***}p < 0.001$ level. ABA, abscisic acid; KD, knocked down; OE, overexpressing; WT, wild type [Color figure can be viewed at wileyonlinelibrary.com]

are obtained by aquaporin gene expression modification at the whole plant level under controlled growth conditions (Chaumont & Tyerman, 2014; Maurel et al., 2016). In accordance with these observations, the daily mean transpiration rate of the PIP2;5 OE lines increased by 14%–22% and decreased by 9% in *pip*2;5 KD lines in comparison with the WT transpiration in well-watered soil. This change in transpiration was possibly due to the regulation of the tissue hydraulic properties mediated by aquaporins in roots and/or shoots (Ding et al., 2020; Pantin et al., 2013; Prado et al., 2013; Shatil-Cohen et al., 2011). Sade et al. (2014) found that g_s increased in *Arabidopsis* lines overexpressing NtAQP1 (a PIP1 isoform) under the control of 35S promoter or the main photosynthetic tissue promoter *FBPase*, but not when the expression was controlled by the stomatal specific *KST1* promoter. Conversely, transpiration and g_s were decreased by constitutively silencing PIP1s, but not by specific silencing in bundle sheath cells (Sade et al., 2014). Altogether, these data indicate that aquaporins can indirectly affect g_s through changes in tissue hydraulic properties affecting signalling processes.

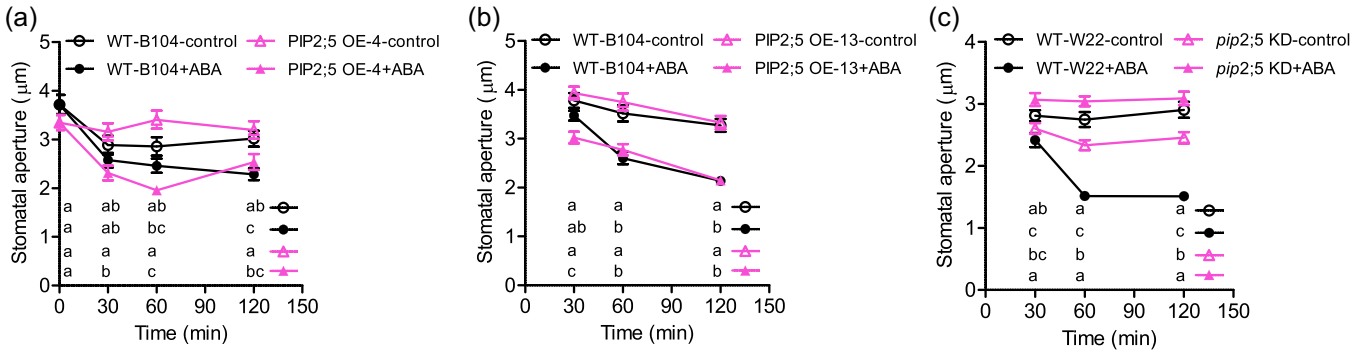

FIGURE 6 Stomatal aperture after ABA treatments (experiment 4, Table 1). (a) PIP2;5 OE-4, (b) PIP2;5 OE-13 and (c) *pip2;5* KD. The epidermal peels were incubated in 10 μM ABA for 30, 60 and 120 min, and the stomatal aperture was measured from $n > 40$ stomata in two independent experiments. Data are expressed as the mean ± SE. One-way ANOVA with Tukey's posttest was applied to detect stomatal aperture difference significance at the $p < 0.05$ level between WT and the PIP2;5 deregulated maize lines and the effect of ABA treatments. The statistical analysis was performed separately for each time point. A significant difference is indicated by different letters in the figure. ABA, abscisic acid; ANOVA, analysis of variance; KD, knocked down; OE, overexpressing; WT, wild type [Color figure can be viewed at wileyonlinelibrary.com]

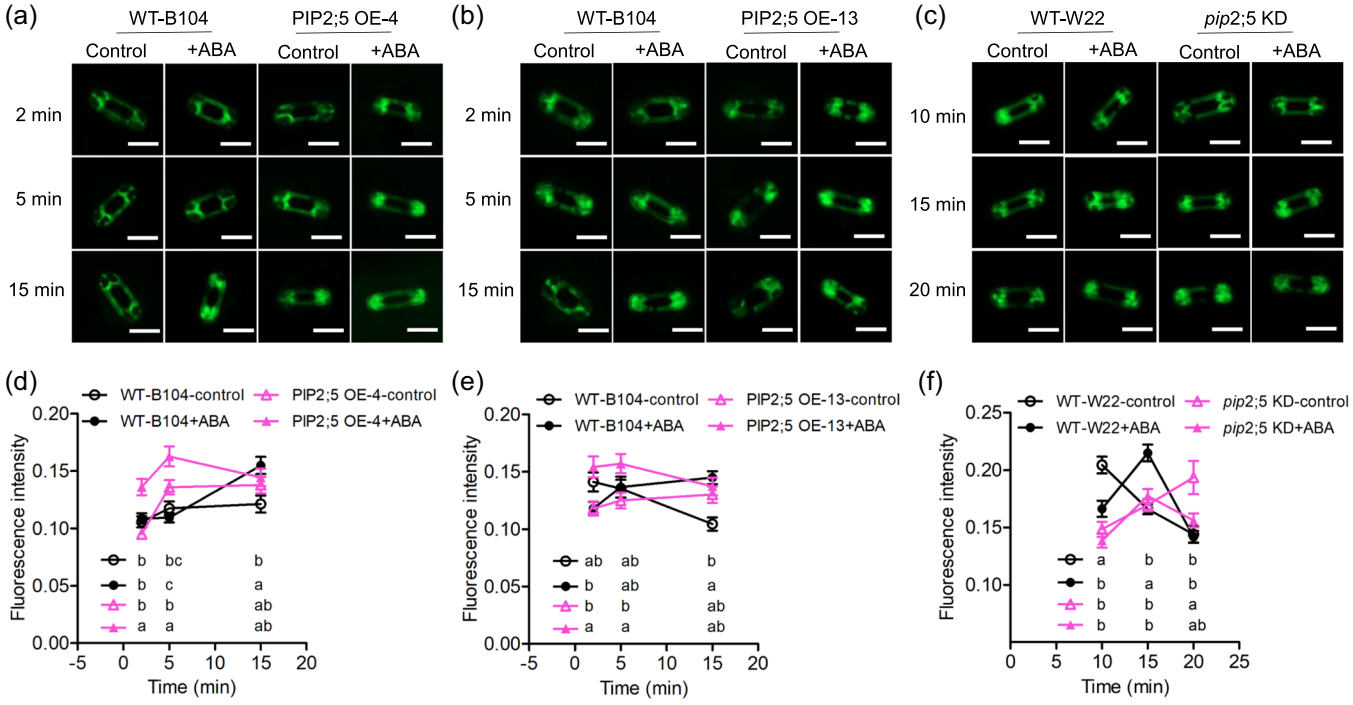

FIGURE 7 ROS accumulation in guard cells after ABA treatments (experiment 4, Table 1). (a,d) PIP2;5 OE-4, (b,e) PIP2;5 OE-13 and (c,f) *pip2;5* KD. (a–c) ROS signal in stomatal complexes. (d–f) Quantification of the time course of ROS signal under ABA treatments. The epidermal peels were incubated in 10 μM ABA at the indicated time, and the H_2DCFDA fluorescent signal was detected. The data were calculated from $n > 50$ guard cells in two to three independent experiments. One-way ANOVA with Tukey's posttest was applied to determine the stomatal aperture difference significance at the $p < 0.05$ level between WT and the PIP2;5 deregulated maize lines and the effect of ABA treatments. The statistical analysis was performed separately for each time point. The significant difference is indicated by different letters in (d–f). Scale bars = 20 μm in (a–c). ABA, abscisic acid; ANOVA, analysis of variance; KD, knocked down; OE, overexpressing; ROS, reactive oxygen species; WT, wild type [Color figure can be viewed at wileyonlinelibrary.com]

In soil water deficit conditions, the decrease in transpiration and g_s results from ABA-induced stomatal closure (Cai et al., 2017; Zhu, 2002). Here, we showed that, in the PIP2;5 OE lines, the transpiration rate was less in a mild water deficit condition (−0.1/−0.2 MPa) and g_s decreased more rapidly after low ABA concentration (0.1 μM) treatment than in WTs. Interestingly, an inverse behaviour was observed for the *pip2;5* KD plants. These results indicate that PIP2;5 is involved in regulating stomatal closure under mild drought stress or ABA treatments.

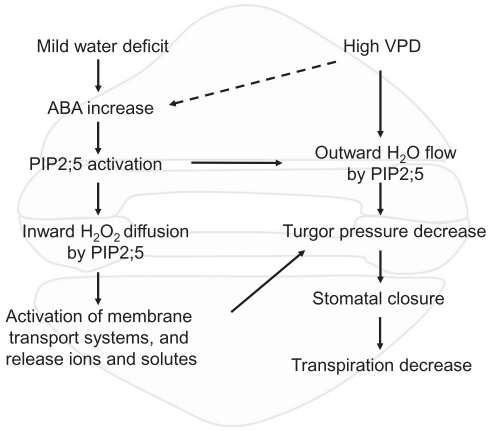

FIGURE 8 Model explaining PIP2;5 roles in regulating stomatal closure and transpiration decrease upon mild water deficit and high vapour pressure deficit

Overexpressing *Vicia faba Vf*PIP1 in *Arabidopsis* (Cui et al., 2008) or the apple MdPIP1;3 in tomato plants (L. Wang et al., 2017) also results in faster stomatal closure in transgenic plants than in controls upon drought stress or ABA treatments. However, we showed that this phenotype was dependent on the water deficit conditions. The difference in stomatal closure behaviour was only observed under mild soil water deficit or low concentration ABA treatment, but not under severe water deficit or high ABA treatments, suggesting that stomatal closure is regulated by several factors under drought stress or ABA treatment, possibly involving signalling events both in stomatal complexes and in vascular bundle sheath (Pantin et al., 2013; Shatil-Cohen et al., 2011). In severe soil water deficit conditions ($\Psi_{soil} \approx -0.30$ to−0.50 MPa), PIP2;5 OE maize lines may maintain root water uptake ability and leaf hydraulic conductance (Ding et al., 2020), resulting in similar or even higher plant transpiration and growth as in WT plants. Altogether, these data indicate that the effect of PIP2;5 overexpression on maize plant growth and water relations is really dependent on water stress conditions and that, in mild water deficit, PIP2;5 OE lines perform better than control plants by faster stomatal closing and/or increased root water uptake ability, as previously observed for plants overexpressing other PIP aquaporins (Groszmann et al., 2017; Sade & Moshelion, 2017).

Additionally, we found that aquaporin affects transpiration/g_s depending on VPD. High VPD-induced stomatal closure depends on either active ABA signalling or passive hydraulic regulation in guard cells (Pantin & Blatt, 2018), and a recent study indicates that hydraulic regulation mainly controls the stomatal movement in *Arabidopsis* (Merilo et al., 2018) and in maize (Devi & Reddy, 2020). We propose that PIP2;5 OE guard cells lose water faster upon high VPD due to the presence of active PIP2;5 aquaporins in the plasma membrane of guard cells and/or subsidiary cells. Indeed, it is thought that active water channels accelerate the diffusion of water across the membrane, thus leading to a faster guard cell turgor pressure decrease, stomatal closure and lower plant transpiration (Ding et al., 2020).

4.2 | ABA-mediated stomatal closure depends on stomatal expressed PIP2;5

Stomatal aperture and closure depend on signalling molecules such as ABA, H_2O_2 and CO_2, and molecular events, leading to variations in guard cell turgor pressure, and thus on water fluxes across their plasma membrane. Due to their multiple channel specificity, aquaporins are thought to contribute to different stomatal movement processes (Chen et al., 2017; Heinen et al., 2014; Nunes et al., 2020). The first functional evidence of an aquaporin in the stomatal movement was provided by studies using epidermal peels from *Arabidopsis Atpip2;1* KO plants. AtPIP2;1 was required for ABA-dependent stomatal closure and, during this process, phosphorylation of AtPIP2;1 by the specific kinase OST1 activated water and H_2O_2 transport (Grondin et al., 2015; Rodrigues et al., 2017). We found that PIP2;5 OE stomata from epidermal peels closed faster upon ABA treatment, while stomatal closure was insensitive to ABA treatment in *pip2;5* KD plants. This faster stomatal closure was correlated with ROS accumulation (H_2O_2) in PIP2;5 OE guard cells. Actually, H_2O_2 is a key factor in ABA and CO_2 signalling regulating stomatal closure (Chater et al., 2015; Rodrigues et al., 2017), and H_2O_2, produced in the apoplasm by the activated NADPH oxidases, acts in guard cells as a Ca^{2+} channel activity regulator, leading to SLAC1 activation in the plasma membrane (Pei et al., 2000) and stomatal closure. Several aquaporins, including maize PIP2;5, were previously shown to facilitate H_2O_2 diffusion when expressed in yeast (Bienert et al., 2007, 2014) or in planta (Rodrigues et al., 2017; Tian et al., 2016). Altogether, these data strongly suggest that PIP2;5 expressed in maize guard cells acts as H_2O_2 channels.

Recently, an *Arabidopsis* quadruple *Atpip1;1*, *Atpip1;2*, *Atpip2;1* and *Atpip2;2* mutant was generated but did not exhibit any significant difference in stomatal aperture or g_s upon 2 µM ABA treatment compared with the WT (Ceciliato et al., 2019). The same group was also unable to confirm the stomatal ABA insensitivity of *Atpip2;1* mutant plants (C. Wang et al., 2016). However, these conflicting results might be explained by different plant growth conditions and/or experimental methods. For example, in our study, the decrease or increase in g_s observed in PIP2;5 OE plants and *pip2;5* KD plants, respectively, were only recorded using 0.1 µM ABA treatments, but not with 0.5 µM or greater ABA concentrations. This indicated that stomatal closure is due to ABA signalling in guard cells when the epidermis is investigated, while stomatal closure is regulated by ABA signalling in both guard cells and vascular bundle sheath cells in intact leaves (Pantin et al., 2013). We showed that PIP2;5 OE guard cells are sensitive to mild soil water deficit or low ABA concentration. However, under severe water deficit or high ABA treatment, stomatal closure might be more regulated by ABA signalling in vascular bundle sheath cells, as demonstrated in *Arabidopsis* by the fact that high ABA (50 µM) treatment was able to decrease stomatal aperture of ABA insensitive mutants (*ost2-1*, *abi1-1* and *slac1-1*), through leaf hydraulic conductance regulation (Pantin et al., 2013).

5 | CONCLUSION

Plants close stomata and decrease transpiration for water conservation under water deficit environments. Manipulation of the dynamic of stomatal aperture/conductance could be an interesting strategy to increase water use efficiency, as well as the drought resilience (Ding & Chaumont, 2020; Papanatsiou et al., 2019). Here, we demonstrated that PIP2;5 expressed in maize stomatal complexes regulates stomatal closure sensitivity to mild water deficit and high VPD, leading to a lower transpiration rate. In our model described in Figure 8, we proposed that, after sensing water deficit signals, plants can react by increasing ABA concentration in the guard cells, leading to an activation of PIP2;5 present in the plasma membrane. Due to the PIP2;5 H_2O_2 channel activity, an inward H_2O_2 diffusion contributes to its accumulation in guard cells, activating the membrane transport system releasing ions and solutes. These molecular signalling events result in an outward water flow facilitated by aquaporins including PIP2;5, a decrease in turgor pressure and stomatal closure. Besides, high VPD might directly regulate the outward water flow through PIP2;5 and hence lead to stomatal closure. Consequently, transpiration decrease is more sensitive to water deficit or high VPD. In conclusion, PIP2;5 represents an important player of maize stomatal movement dynamics. It improves ABA signalling sensitivity probably by facilitating H_2O_2 accumulation and speeding up stomatal closure. This process is beneficial to plant growth in mild water deficit, allowing water conservation and a greater leaf elongation rate (Ding et al., 2020).

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

Data and materials are available on request from the corresponding author.

ORCID

Lei Ding http://orcid.org/0000-0003-1262-5289
Boris Parent http://orcid.org/0000-0002-7600-9592
François Tardieu http://orcid.org/0000-0002-7287-0094
François Chaumont http://orcid.org/0000-0003-0155-7778

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