

AtPIP2;5 Aquaporin Affects Mesophyll Conductance to CO₂ and Stomatal Density in *Nicotiana tabacum* Leaves

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Abstract: The plasma membrane intrinsic protein AtPIP2;5 aquaporin is known for accelerating water flow into plant cells; however, its role in CO₂ transport in leaf mesophyll cells (as a 'cooporin') is not well understood. We investigated parameters of leaf water loss and CO₂ uptake in transgenic tobacco (*Nicotiana tabacum* cv. Xanthi) leaves expressing the AtPIP2;5 gene from *Arabidopsis thaliana* (AtPIP2;5) and in wild-type (WT) tobacco. The control and transgenic plants were subjected to osmotically induced drought stress and a recovery period. Transpiration and photosynthesis rates (E , A_N), stomatal and mesophyll conductance to CO₂ (g_s , g_m), water use efficiency (WUE) and stomatal density (SD) were measured using gas exchange and stable isotope techniques. AtPIP2;5 expression in well-watered plants did not change A_N and g_s but increased g_m by 42% compared to WT ($p \leq 0.05$). After 7 days of drought, g_m decreased to 50% of its original value in PIP2;5 plants while remaining almost unchanged in the controls. A_N also decreased significantly in stressed PIP2;5 plants compared to WT, while g_s did not differ between genotypes. g_m did not respond to 7 days of recovery in PIP2;5 and WT. However, in the transgenic plants, we found significant increases in SD and SI compared to WT. Instantaneous WUE was lower in transgenics than in WT. Our results suggest that AtPIP2;5 expressed in tobacco is also involved in CO₂ transport in mesophyll cells during photosynthetic CO₂ fixation. However, its facilitating role may turn into inhibition of CO₂ flux during drought stress, persisting even after recovery.

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Keywords: Aquaporin, AtPIP2;5, Mesophyll conductance (g_m), Water use efficiency (WUE)

Introduction

Water deficit is one of the main limiting factors in plant growth and survival. Effective water uptake and transport is important not only for plant growth under well-watered conditions but especially for the ability of a plant to tolerate adverse environmental conditions [1-3]. Therefore, a broad scale of physiological and anatomical adaptations and survival strategies has developed to control plant water status. Among those are large families of aquaporins, water channel proteins encoded by specific genes, which are divided into four different subfamilies based on subcellular localization and sequence similarity: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like MIPs or NOD26-like intrinsic proteins (NIPs), and small basic intrinsic proteins (SIPs) [4-6]. The presence of aquaporin in a membrane can enhance the water transport activity and,

thus, can be regarded as a specific adaptation for facilitating recovery after drought stress [7]. The water transport activity of aquaporin has been verified both *in vitro*, using a *Xenopus laevis* oocyte system, and *in vivo*, via the expression of an antisense construct of PIPs or PIPs expression in *Arabidopsis thaliana* [8,9] and tobacco [10]. Understanding the function of plasma membrane intrinsic protein 2;5 (AtPIP2;5) in *Arabidopsis* and its impact under stress conditions can shed new light on plant water relations [7,11,12].

Water use efficiency (WUE) is a canopy, plant or leaf trait used to estimate the amount of carbon gain per unit of taken up and, finally, lost water. Plant or leaf WUE integrated over the growth period can be estimated from stable carbon isotope composition ($\delta^{13}\text{C}$) of leaf biomass. Carbon isotope discrimination ($\Delta^{13}\text{C}$), calculated from the difference between atmospheric and plant $\delta^{13}\text{C}$, is negatively correlated with intrinsic water-use efficiency [13,14]. Aquaporins can play a dual role in affecting WUE. First, they may accelerate uptake and affect flow of water through the plant body including leaves [15] and second, they may also boost CO_2 access to chloroplasts via CO_2 -specific channeling activity at the plasma membrane [16,17]. Leaf internal CO_2 transport to chloroplasts in mesophyll cells has been recognized as one of the rate-limiting steps of photosynthesis. The diffusive properties of the pathway are characterized by the mesophyll conductance (g_m). Recently, some authors demonstrated that aquaporins could be involved in regulation of g_m [18-20] and consequently, of photosynthesis rate and WUE. In addition to short-term variations in stomatal aperture, stomatal density (SD) and guard cell morphology are leaf traits affecting water use efficiency of plants [21,22]. Aquaporins are involved in leaf development and can be linked to higher SD in *AtPIP1* expressing tobacco plants [15]. In turn, alterations of SD are associated with alterations in photosynthesis rate, which is important for plant growth and development (e.g., [23,24]).

Although the number of reports demonstrating the function of AtPIP2;5 aquaporins in regulating plant water status under stress conditions is expanding, the roles of AtPIP2;5 in the modulation of stomatal frequency and mesophyll conductance for CO_2 , respectively, for plant recovery from water shortage are not fully understood. Moreover, the potential relationship between the role of aquaporins in the regulation of water status and the regulation of stomatal behavior is unclear. In this study, we aimed to investigate how the expression of *AtPIP2;5* contributes to the control of water loss and carbon gain in terms of water use efficiency, SD as well as CO_2 mesophyll conductance during treatment with osmotic stress and recovery from stress.

Results

As drought stress induced a more severe water deficiency in *AtPIP2;5* expressing tobacco plants compared with the wild-type [7], we expected that photosynthetic parameters of our transgenic plants under drought stress would deteriorate more strongly than in the wild-type. Based on the experimental workflow shown in Fig. 1, the physiological and anatomical responses of AtPIP2;5-expressing tobacco plants to drought stress and subsequent recovery were systematically evaluated. Gas exchange measurements did not show any differences in net photosynthesis (A_N) and stomatal conductance (g_s) between the wild-type and AtPIP2;5 under well-watered conditions (Fig. 2A, B). Mesophyll conductance (g_m) was significantly higher in well-watered AtPIP2;5 expressing plants than in the wild-type. As expected, A_N in AtPIP2;5 expressing plants decreased dramatically after 7 days of drought stress to significantly lower values than in stressed wild-type plants; these values were also significantly lower than those after recovery in all plants. A_N under osmotic stress and after recovery was significantly lower in AtPIP2;5 expressing than in wild-type plants. g_s did not differ significantly between both genotypes, irrespective of treatment (Fig. 2B). While g_m in AtPIP2;5 expressing plants noticeably decreased under osmotic stress and remained low after recovery, in wild-type plants g_m was similar in all treatments.

In order to determine the effect of aquaporins on leaf water economy under osmotic stress, WUE of the leaf was determined in wild-type and transgenic plants. In well-watered and recovered plants, we compared intrinsic WUE (WUEi), as inferred from $\Delta^{13}\text{C}$ of leaf dry matter. No significant differences in WUEi were observed between the wild-type and AtPIP2;5 expressing plants

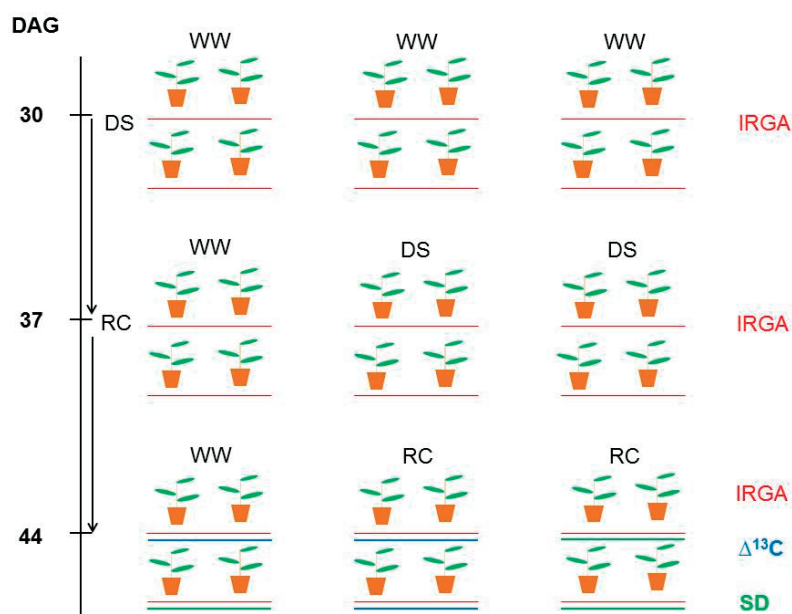


Fig. 1. Schematic of the experimental design. Abbreviations denote: days after germination (DAG), well-watered (WW), drought stressed (DS), recovered (RC). IRGA: time of gas exchange measurement, SD: time of stomatal density determination, $\Delta^{13}\text{C}$: time of leaf dry mass sampling for ^{13}C discrimination analyses. This design applies to control and transgenic plants.

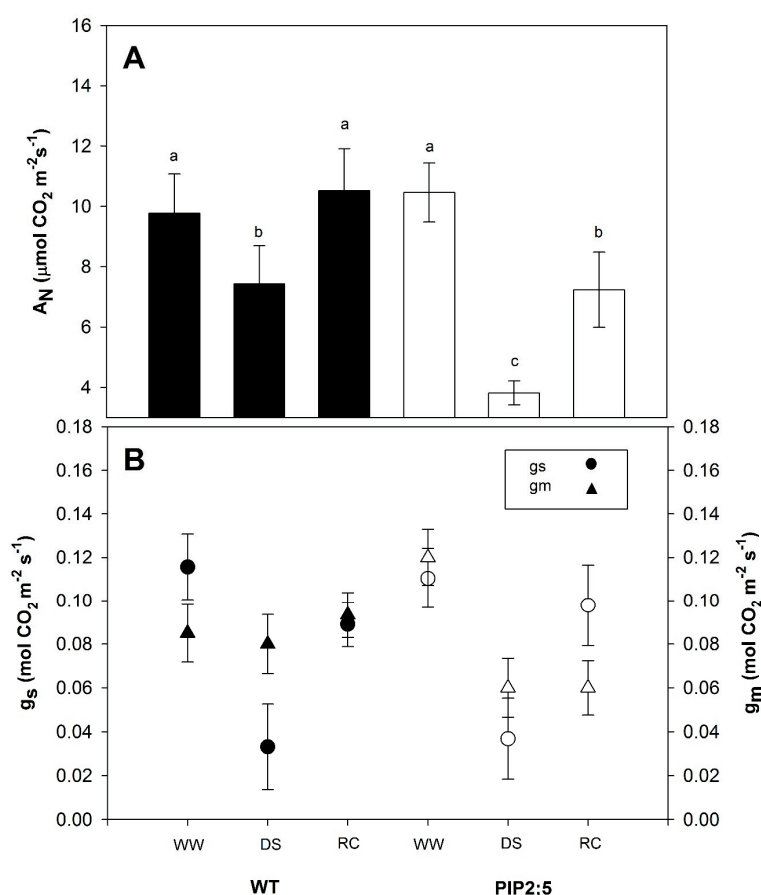


Fig. 2. Effects of drought stress on gas exchange parameters in wild-type (closed symbols) and *AtPIP2;5* expressing tobacco plants (open symbols). Net photosynthesis rate (A_N) (panel A), stomatal conductance (g_s) (circles) and mesophyll conductance (g_m) (triangles) (panel B) of well-watered control (WW), drought-stressed (DS) and recovered (RC) plants. Error bars represent standard deviation from three independent experiments ($9 \leq n \leq 12$). Different letters denote values that are statistically different.

under well-watered conditions, and the same was true after the stress and recovery periods (Fig. 3A). Instantaneous WUE was calculated as the A_N/E ratio obtained from gas exchange measurements. It increased dramatically in the control plants subjected to osmotic stress, while in *AtPIP2;5* plants it remained almost unaffected (Fig. 3B). WUE after stress recovery was higher by a factor of 2.4 in WT than in the transgenics. These results indicate that *AtPIP2;5* expression reduced instantaneous WUE during drought stress and recovery primarily through decreased in net photosynthesis (A_N) than through increased transpirational water loss, as evidenced by the significantly lower A_N in transgenic plants (Fig. 2A) despite similar transpiration rates between genotypes. SD, pavement cell density (PCD) and stomatal index (SI) were analyzed in leaves of *AtPIP2;5* expressing and wild-type tobacco plants. To avoid interference of lateral heterogeneity of epidermal traits, we collected leaf discs from the area enclosed in the LI-6400 leaf chamber after the gas exchange measurements had been finished. SD was estimated from the leaf imprints taken on the abaxial side of the discs. In both genotypes, similar values of SD were detected under well-watered conditions (Fig. 4). After 7 days of drought stress and 7 days of recovery, SD and SI significantly increased in *AtPIP2;5* expressing plants while they remained steady in the wild-type (Fig. 4A, C). These anatomical changes in stomatal and pavement cell distribution patterns indicate developmental responses in epidermal patterning associated with *AtPIP2;5* expression.

Since *AtPIP2;5* expressing leaves showed significantly increased SD after osmotic stress and recovery, we investigated the rela-

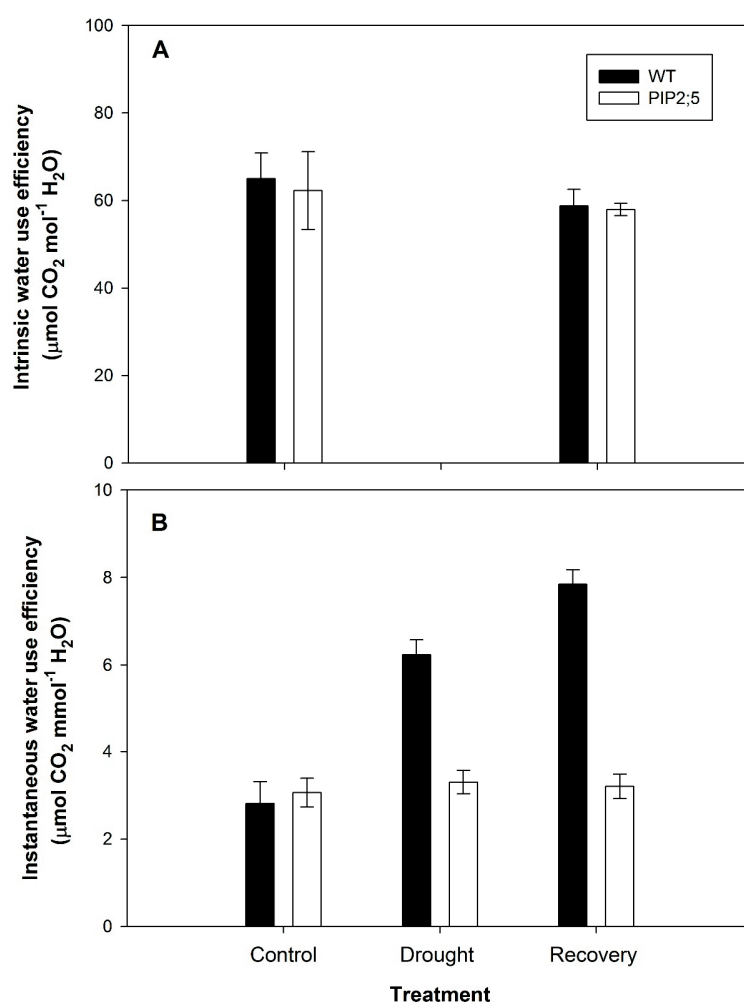


Fig. 3. Water use efficiency (WUE) of WT and *AtPIP2;5* expressing tobacco plants. Intrinsic water use efficiency as inferred from $\delta^{13}\text{C}$ in leaf biomass (A) or instantaneous WUE estimated from gas exchange measurements (B) of wild-type (closed symbols) and *AtPIP2;5* expressing plants (open symbols). The leaves for $\delta^{13}\text{C}$ analyses were collected at the end of the experiments after a 7-day drought stress treatment followed by a 7-day recovery period. The instantaneous WUE was computed as the A_N/E ratio. Error bars represent standard deviations ($9 \leq n \leq 12$).

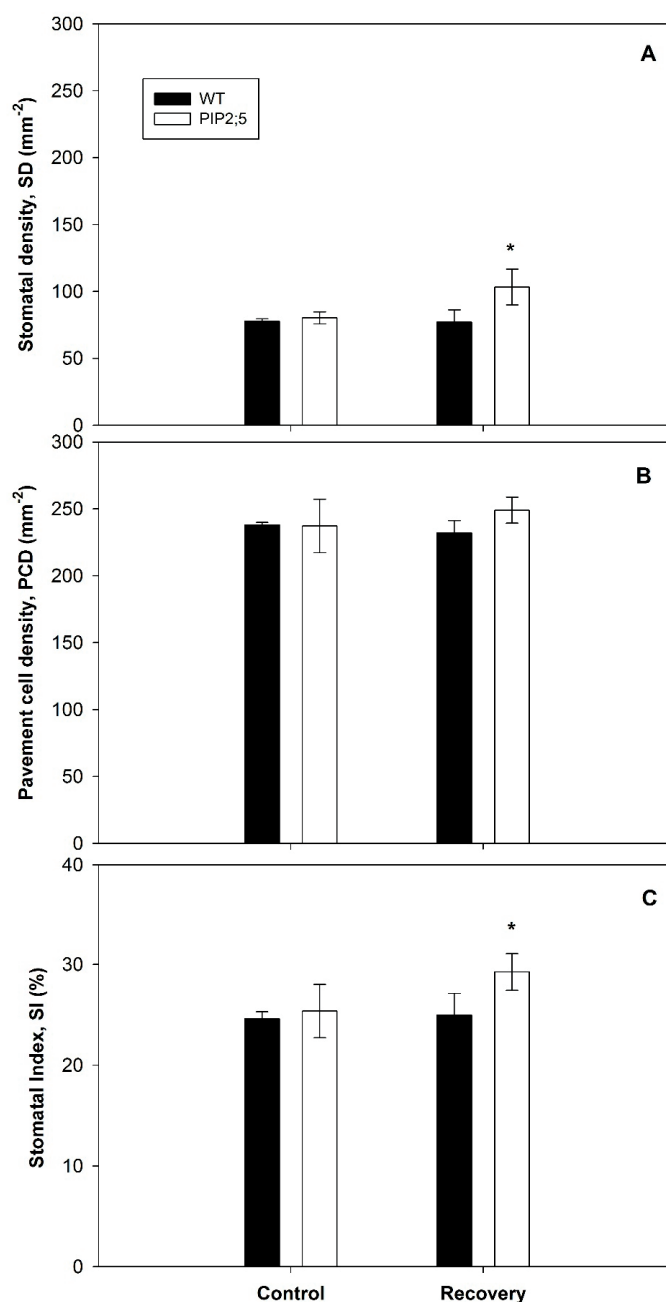


Fig. 4. The effect of osmotic stress and recovery on stomatal traits of tobacco expressing *AtPIP2;5* and relevant wild-type. The effect of 7 days of osmotic stress followed by 7 days of recovery on stomatal density (SD) (panel A), pavement cell density (PCD) (panel B) and stomatal index (SI) (panel C) of fully expanded young leaves of wild-type (closed bars) and *AtPIP2;5* expressing leaves of tobacco (open bars). Asterisks indicate significant differences ($p \leq 0.05$) between controls (well-watered throughout) and plants subjected to drought stress and recovery.

tionship between SD and the photosynthetic parameters. When subjected to drought stress, values of A_N , g_s and transpiration rate (E) decreased in both genotypes. Although differences between the wild-type and *AtPIP2;5* expressing plants were detected, they were usually small and insignificant except for the *AtPIP2;5*-increased g_m in well-watered and *AtPIP2;5*-reduced A_N and g_m in osmotically-stressed plants. After recovery, SD and g_s as well as E increased with expression of *AtPIP2;5* (Fig. 5A, B). Increased SD in *AtPIP2;5* expressing plants probably contributed to higher g_s and E and lower instantaneous WUE after recovery compared to recovered wild-type plants (Fig. 3B). However, intrinsic WUEi remained unaffected by elevated SD (Fig. 5C), indicating that the SD increase enhanced total gas exchange without altering the efficiency of individual stomata. The increased SD

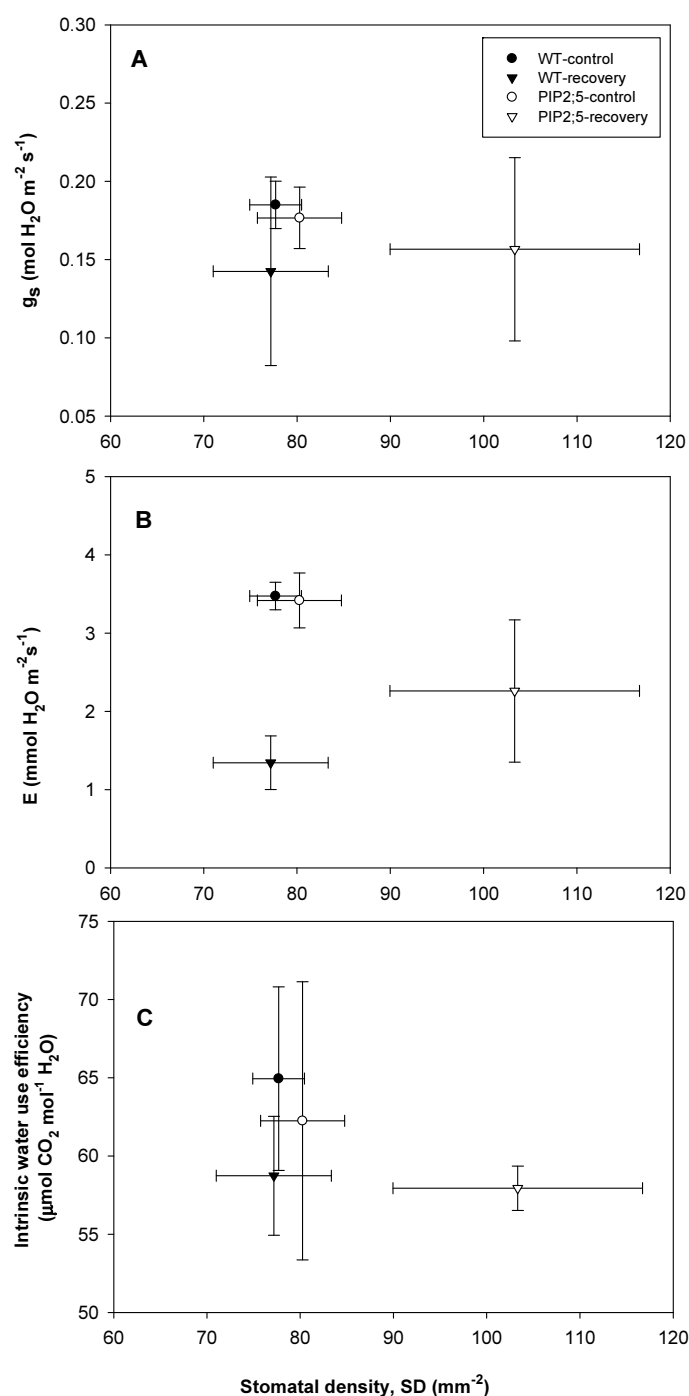


Fig. 5. Relationships between stomatal density and gas exchange parameters of tobacco expressing *AtPIP2;5* and its wild-type. The relationships between stomatal density (SD) and stomatal conductance (g_s) (panel A), transpiration rate (E) (panel B) and intrinsic water use efficiency (panel C). Each symbol represents the mean for well-watered (circles) or stressed and recovered (triangles) plants for wild-type (closed symbols) or transgenics (open symbols). Error bars represent standard deviation for nine plants in three independent experiments.

in *AtPIP2;5* expressing leaves following osmotic stress and recovery might be expected to facilitate the recovery of A_N to pre-stress values by enhancing CO₂ uptake. However, despite the elevated SD and consequent increase in intercellular CO₂ concentration (C_i , Fig. 6), the pre-stress value of A_N was not restored in transgenic plants. This observation suggests that the limitation to photosynthetic recovery in *AtPIP2;5* plants was not at the stomatal level (CO₂ entry into leaves) but rather at the mesophyll level (CO₂ transport to chloroplasts). The persistently reduced mesophyll conductance (g_m) in stressed and recovered *AtPIP2;5*

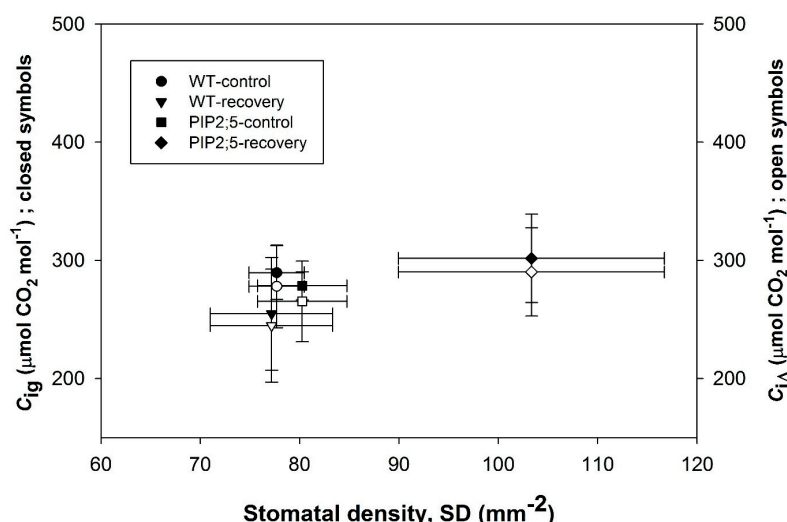


Fig. 6. The relationship between intercellular CO₂ concentration (C_i) and stomatal density on the abaxial leaf side of tobacco expressing *AtPIP2;5* and its wild-type. Each symbol is the mean value of C_i calculated from gas exchange (C_{ig} , closed symbols) or C_i inferred from $\Delta^{13}\text{C}$ in leaf dry matter ($C_{i\Delta}$, open symbols). Error bars represent standard deviation for nine plants in three independent experiments.

plants (Fig. 2B) likely prevented efficient CO₂ delivery to Rubisco, thereby constraining A_N despite adequate leaf internal CO₂ concentration (C_i). Thus, the increased SD appears to represent a compensatory developmental response to low chloroplastic CO₂ (C_c) rather than an effective mechanism for restoring photosynthetic capacity. These observations indicate that A_N in *AtPIP2;5* expressing plants is facilitated by increased g_m under well-watered conditions but under stress and even after recovery g_m falls, and in spite of the greater frequency of stomata A_N remains lower than in the wild-type.

Discussion

In this study, we focus on the function of the *AtPIP2;5* gene expressed in tobacco plants in the control of water use and carbon assimilation during drought stress and plant recovery. Recently, it was shown that *AtPIP2;5* is involved in the facilitation of water transport across plasma membranes [12]. Here, we wanted to test the possibility of a dual function of this protein, i.e., its involvement also in CO₂ transport from the intercellular air space into chloroplasts. If *AtPIP2;5* facilitates the flux of CO₂, an increase in mesophyll conductance to CO₂ (g_m) at enhanced expression levels of *AtPIP2;5* may be expected. The role of NtAQP1 aquaporin in g_m regulation in transgenic tobacco plants has been demonstrated by [18]. NtAQP1 facilitated CO₂ flux when expressed in yeast cells, but the PIP2 aquaporin from tobacco, NtPIP2;1, did not have this effect [25]. Nevertheless, *AtPIP2;5* is one of the candidate proteins whose CO₂ permeability was indicated by the alignment of amino acid sequences of PIP1s and PIP2s at the corresponding region of *Arabidopsis* and barley and, very recently, PIP2 aquaporins were suggested to facilitate CO₂ transport [26]. Here, we have found that g_m in *AtPIP2;5* expressing tobacco leaves tended to be higher than wild-type under well-watered conditions (Fig. 2B), though the difference was not statistically robust. This result supports the possible mechanism discussed by [26] although the increase in g_m of *AtPIP2;5* plants was not statistically significant. Further analyses with *AtPIP2;5*, such as a direct measurement of CO₂ transport ability [20,26], are needed to completely understand the function of *AtPIP2;5* in CO₂ transport across mesophyll cells.

[15] reported that expression of *AtPIP1* in tobacco plants enhanced SD under favorable conditions. Several reports have shown that SD and/or SI increase under water stress [27–29]. In the present study, the expression of *AtPIP2;5* did not affect SD in tobacco plants under well-watered conditions. During osmotic stress and the following recovery period, SD and SI of *AtPIP2;5*

expressing tobacco leaves increased significantly (by approximately 20%, $p \leq 0.05$) compared with the wild-type. The increased SD and SI observed in *AtPIP2;5*-expressing plants under osmotic stress indicate a coordinated developmental response rather than a simple anatomical change. Because *AtPIP2;5* is constitutively expressed, its influence on epidermal development operates continuously during leaf expansion. The stress-induced reduction in g_m reduced chloroplastic CO_2 concentration (C_c), and recent evidence suggests that C_c —rather than intercellular CO_2 (C_i)—serves as the primary signal regulating stomatal development [31]. Thus, the sustained low C_c likely triggered enhanced stomatal differentiation, resulting in higher SD and SI. This interpretation is consistent with reports showing that stomatal development is negatively regulated by CO_2 availability during leaf expansion across multiple species [31]. As these newly formed stomata became functional during recovery, greater stomatal conductance increased CO_2 influx and elevated C_i , even though g_m and C_c remained low. This physiological uncoupling—elevated C_i but persistently low C_c —reflects enhanced stomatal CO_2 entry that exceeds the mesophyll's diminished transport capacity. Although g_m appeared to increase slightly after rewatering, statistical analyses confirmed that its recovery was not significant. Overall, these results indicate that *AtPIP2;5* enhances g_m under favorable conditions but becomes functionally impaired under stress, eliciting compensatory adjustments in stomatal development that increase SD and SI [28,32,33] without restoring chloroplastic CO_2 supply or photosynthetic performance.

One of the objectives of the present study was to test whether the expression of *AtPIP2;5* may contribute to controlling water use and carbon economy of a leaf during its recovery from drought stress. Intrinsic WUE is often estimated by analyzing stable isotope composition of plant biomass although it should be used with caution [14]. To determine instantaneous WUE during osmotic stress, photosynthesis and transpiration rates A_N and E were employed. Tobacco leaves expressing *AtPIP2;5* showed almost invariable values of both intrinsic and instantaneous WUE under drought stress and recovery. Increased SD in *AtPIP2;5* plants co-varied positively with E (Fig. 5B). What might explain the rather complex syndrome of *AtPIP2;5* expression? It seems that a non-negligible part of CO_2 reaching the chloroplast stroma passes through aquaporins and this part is enhanced when *AtPIP2;5* is expressed. During drought stress however, ABA or another concomitant factor probably disables *AtPIP2;5* for CO_2 transport, i.e. reduces mesophyll conductance, and lowers CO_2 concentration in chloroplasts and photosynthesis rate dramatically. This may stimulate the fraction of epidermal cells which become guard cells (greater SD and SI) and increase transpiration rate. Water use efficiency is therefore reduced in *AtPIP2;5* expressing plants exposed to drought; however, transpiration rate and leaf cooling are enhanced. The latter feature may help the *AtPIP2;5* expressing plants cope with short-term drought stress.

In conclusion, we have shown that expression of *AtPIP2;5* in tobacco leaves did not affect water consumption (transpiration rate and stomatal conductance) in well-watered plants but increased (although non-significantly) mesophyll conductance to CO_2 and photosynthesis rate. However, when the *AtPIP2;5* expressing plants were exposed to drought and recovery periods, g_m dropped markedly and SD, SI and transpiration rate significantly increased compared to WT. Our results suggest that *AtPIP2;5* expression does not directly affect stomatal conductance under well-watered conditions (Fig. 2B), but primarily influences g_m to CO_2 , thereby affecting CO_2 access to chloroplasts. The observed increase in g_s during recovery in *AtPIP2;5* plants (Fig. 5B) was a secondary consequence of increased SD (Fig. 4A) rather than a direct effect of *AtPIP2;5* on guard cell function. When challenged by water shortage, the primary impact of *AtPIP2;5* expression is on mesophyll CO_2 transport capacity, with effects on stomatal development and density emerging as a longer-term developmental response to altered CO_2 availability in chloroplasts. The increased SD observed in transgenic lines may reflect a secondary developmental response. The mechanistic links between *AtPIP2;5* expression, g_m and drought stress remain to be revealed.

Materials and Methods

Plant material, growth conditions and treatments

Tobacco (*Nicotiana tabacum* cv. Xanthi) plants (wild-type) and tobacco plants expressing *AtPIP2;5* [7] were used for this

study. The seeds were sown on half-strength Murashige and Skoog [34] medium and grown at $23\pm 2^\circ\text{C}$ under a long day condition (16 h photoperiod). To avoid developmental differences, 12 selected seedlings of uniform appearance were transferred to pots 10 days after germination (DAG) and grown in a 2:1:1 mixture of vermiculite, peat moss and perlite. Plants were grown in a glasshouse under natural light [midday average over all photoperiods during the experiment was $600\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ (PAR)], 16/8 h (day/night) photoperiod, temperature of $25\pm 2^\circ\text{C}$, and air humidity of 40-50%. The plants were kept well-watered with daily irrigation. A nutrient solution [35] was added once a week. At 30 DAG, both groups of 12 selected tobacco or transgenic plants were divided into three subgroups (Fig. 1). Four plants were maintained well-watered until the end of the experiment: leaf imprints were collected to calculate SD and leaf dry mass was sampled for carbon isotope analyses. The remaining eight plants were subjected to drought stress for the next 7 days by applying nutrient solution supplemented with 200 mM mannitol. After 7 days, the stress treatment was relieved, the mannitol-containing solution exchanged with pure nutrient solution and the experiment continued for another 7 days. Simultaneous gas exchange and ^{13}C discrimination measurements were performed in all plants. The treated plants were divided into two subgroups and used to collect leaf imprints or leaf dry mass for $\delta^{13}\text{C}$ analyses, respectively.

Leaf gas-exchange and carbon isotope discrimination measurements: estimation of mesophyll conductance

Gas-exchange parameters (transpiration and photosynthesis rates, stomatal conductance) of the first or second leaf of 4 to 6-week-old plants grown under well-watered conditions (controls) or irrigated with 200 mM mannitol were estimated with a portable gas-exchange system (LI-6400, Licor, Nebraska, USA). To estimate the leaf discrimination against $^{13}\text{CO}_2$, a $6\times 2\text{ cm}$ leaf chamber (Li-6400-11) was used to maximize the exposed leaf area and the draw-down in CO_2 between chamber inlet and outlet. The leaf was illuminated with a laboratory-made red and blue LED light source. Net photosynthesis (A_N) and stomatal conductance (g_s) were measured under saturated light (photosynthetic photon flux density PPFD $1500\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ with a 10% fraction of blue light). Leaf temperature was controlled in a narrow range of $25\text{--}26^\circ\text{C}$. The chamber exhaust tube was connected to a 100 ml gas sampling container with a Swagelok Y-piece connection. Under steady-state conditions and at a flow rate through the leaf chamber of $300\ \mu\text{mol (air)}\text{ s}^{-1}$, air leaving the cuvette was collected in the container for 10 min to ensure the air inside the container was exchanged at least 20-25 times [36]. After this period, the chamber exhaust tube was reconnected to the instrument's match valve and the matching procedure of LI-6400 IRGAs was carried out before recording the actual gas-exchange parameters. To collect a reference air sample, the same procedure was carried out with the empty cuvette.

Carbon isotope composition of the air samples was estimated with a continuous-flow stable isotope ratio mass spectrometer (DeltaPlus XL, ThermoFinnigan, Bremen, Germany) coupled via GasBenchII with Precon (both ThermoFinnigan, Bremen, Germany) ensuring CO_2 trapping by the liquid N_2 cooling system. This made it possible to estimate $\delta^{13}\text{C}$ in CO_2 at low as well as high CO_2 concentrations.

Carbon isotope discrimination was calculated according to [37] as:

$$\Delta^{13}\text{C}_{\text{obs}} = [\xi(\delta^{13}\text{C}_{\text{out}} - \delta^{13}\text{C}_{\text{in}}) / (1000 + \delta^{13}\text{C}_{\text{out}} - \xi(\delta^{13}\text{C}_{\text{out}} - \delta^{13}\text{C}_{\text{in}}))] \quad (1)$$

where $\xi = C_{\text{in}} / (C_{\text{in}} - C_{\text{out}})$ and C_{in} and C_{out} are the CO_2 concentrations of the air entering and leaving the leaf chamber.

Mesophyll conductance (g_m) was determined by comparing the observed and predicted discriminations, $\Delta^{13}\text{C}_{\text{obs}}$ and Δ_i , respectively. The predicted discrimination, Δ_i , was calculated according to [38] as

$$\Delta_i = a + (b - a) \times C_i / C_a \quad (2)$$

where a is the fractionation occurring due to diffusion in air (4.4‰), b is the net fractionation by Rubisco and phosphoenolpyr-

uvate carboxylase (PEPC) (29‰), and C_i and C_a are the intercellular and ambient concentrations of CO_2 , respectively.

Finally, g_m was calculated from Eq. (3) [39]

$$\Delta_i - \Delta^{13}C_{obs} = (b - a_w)(A_N/g_m)C_a \quad (3)$$

where a_w is the discrimination due to dissolution and diffusion of CO_2 in water (1.8‰). Fractionation resulting from respiration and photorespiration was assumed to be negligible [40,41].

Estimation of intrinsic WUE by carbon isotope composition

The intrinsic WUE of leaves that had recovered from moderate drought stress was estimated from ^{13}C abundance in leaf dry matter. The first or second leaves were collected at the end of the gas exchange measurements, oven dried at 80°C, ground to a fine powder, packed in tin capsules and oxidized in a stream of pure oxygen by flash combustion at 950°C in the reactor of an elemental analyser (EA, Elementar, vario MICRO cube, Hanau, Germany). After CO_2 separation, the $^{13}C/^{12}C$ ratio (R) was detected via a continuous-flow stable isotope ratio mass spectrometer (IRMS) (Delta plus XL, ThermoFinnigan, Bremen, Germany) connected on-line to the EA. $\delta^{13}C$ in per mill (‰) was calculated as the relative difference between the plant leaf and standard isotope ratios, R: $\delta^{13}C = (R_{plant} / R_{standard} - 1) \times 1000$. VPDB (IAEA, Vienna, Austria) was used as the standard. Cellulose (IAEA-C3) and graphite (USGS 24) were also included in the measurements to ascertain the reliability of the results. Standard deviations of $\delta^{13}C$ estimated in laboratory standard were smaller than 0.2‰. To calculate intrinsic WUE, $\delta^{13}C$ of the leaf dry mass was converted to $\Delta^{13}C$ and entered in the equation.

$$WUE = (C_a/1.6) \times (b - \Delta^{13}C) / (b - a) \quad (4)$$

where the factor 1.6 accounts for the different diffusivities of water and CO_2 in air and $\Delta^{13}C$ was calculated as: $\Delta^{13}C = (\delta_a - \delta_p) / [1 + (\delta_p / 1000)]$ where δ_a and δ_p are $\delta^{13}C$ of ambient air (-8‰) and leaf dry mass, respectively.

Stomatal density (SD)

To estimate SD, the first or second leaves of 6 week old plants were investigated. The nail varnish imprints of abaxial leaf sides were obtained directly from the leaf surface and observed by light microscopy (Olympus BX61). Stomatal and pavement cells were counted on four plants per treatment in 10 randomly selected fields per leaf of 0.13 mm² each. The results were expressed as the average number of stomata or pavement cells per mm² of leaf surface.

Statistical analysis

Three independent experiments involving 36 control and 36 transgenic plants in each were organized. Statistical analyses were performed using R i386 3.0.2 (R for Windows 7, Lucent Technologies) and SigmaPlot v.10.0 (SigmaPlot for Windows, Systat Software, Inc.) at $p=0.05$ confidence level. ANOVAs with Tukey's HSD multiple comparisons were used to analyze all variables.

Data Availability: All data are available in the main text or in the Supplementary Information.

Author Contributions: Rhee, J. performed the experiment and wrote the manuscript.

Notes: The authors declare no conflict of interest

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Additional Information:

Supplementary information The online version contains supplementary material available at <https://doi.org/10.5338/KJEA.2025.44.48>

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