Harpin Hpa1 Interacts with Aquaporin PIP1;4 to Promote the Substrate Transport and Photosynthesis in Arabidopsis

Liang Li1,*, Hao Wang1,*, Jorge Gago2, Haiying Cui3, Zhengjiang Qian4, Naomi Kodama5, Hongtao Ji1, Shan Tian1, Dan Shen1, Yanjuan Chen1, Fengli Sun1, Zhonglan Xia1, Qing Ye4, Wei Sun3, Jaume Flexas2 & Hansong Dong1

Harpin proteins produced by plant-pathogenic Gram-negative bacteria are the venerable player in regulating bacterial virulence and inducing plant growth and defenses. A major gap in these effects is plant sensing linked to cellular responses, and plant sensor for harpin Hpa1 from rice bacterial blight pathogen points to plasma membrane intrinsic protein (PIP). Here we show that Arabidopsis AtPIP1;4 is a plasma membrane sensor of Hpa1 and plays a dual role in plasma membrane permeability of CO2 and H2O. In particular, AtPIP1;4 mediates CO2 transport with a substantial contribute to photosynthesis and further increases this function upon interacting with Hpa1 at the plasma membrane. As a result, leaf photosynthesis rates are increased and the plant growth is enhanced in contrast to the normal process without Hpa1-AtPIP1;4 interaction. Our findings demonstrate the first case that plant sensing of a bacterial harpin protein is connected with photosynthetic physiology to regulate plant growth.

Harpins belong to a unique group of proteins secreted by the type III secretion system in plant-pathogenic Gram-negative bacteria1-3. To date, totally 23 harpins have been identified in different bacterial species and are divided into one-domain and two-domain harpins based on the unitary hydrophilic domain and an additional enzymatic domain1-2. While two-domain harpins potentially associate with the bacterial periplasm or plant cell wall (CW) to facilitate assembly of the secretion machinery, one-domain harpins target plasma membranes (PMs) to cause three distinct biological effects in a variety of plant species4-6. Hpa1 is a one-domain harpin produced by Xanthomonas oryzae pv. oryzae (Xoo), the pathogen that causes bacterial blight of rice Oryza sativa L.2, and performs a full repertoire of functions shared by all harpins tested so far.

One of the biological effects caused by one-domain harpins is the induction of plant immune responses. Harpins represent a special type of microbial patterns, namely invariant microbial epitopes that can be recognized by PM receptors to activate the innate immunity in plants7. After external application to plants or de novo expression in transgenic plants, harpins induce the apoplastic H2O2 signal and its crosstalk with intracellular pathways8 of signaling by phytohormones, such as salicylic acid9 and...
ethylenes. A linkage between apoplastic and cytoplasmic responses has been found in the ability of Hpa1 to stimulate the PM-associated NADPH oxidase and induce apoplastic H2O2, which rapidly moves into cytoplasm to regulate immunity15. The second effect of harpins is to induce plant growth enhancement. Following application or de novo expression, harpins enhance plant growth through cellular transduction of phytohormone signals, such as ethylene10 and gibberellin14. In Arabidopsis, Hpa1-enhanced growth associates with photosynthetic physiology and is attributable to increases of mesophyll conductance (g\text{m}) to CO2 and net photosynthesis (A\text{N}) rate15, indicating the functional linkage of Hpa1 to PIPs that may facilitate CO2 transport16,17. The third effect of some harpins is to serve as type III translocators, which are distinct in nature but function similarly to mediate translocation of type III effectors from bacterial cells into the cytosol of plant cells presumably by recognizing PM sensors2,18. By this mechanism, harpin-type translocators essentially contribute to bacterial virulence to host plants1,2.

Unlike the virulence role that associates with the plant-pathogen interaction process, one-domain harpins induce plant immunity and growth in a pathogen-independent manner. While the immune effect has been extensively studied, molecular mechanisms that govern the role of harpins in plant growth or bacterial virulence is less understood1,2. Pivotal questions are what plant sensors recognize harpins and how they are connected with cellular pathways. Increasing studies point Hpa1 sensors to plant PM-integral proteins. The first 60 amino acids in the 136-residue sequence of Hpa1 are critical for the three biological effects as the N-terminus-deleted version Hpa1\text{ANT} is inactive15,19. In 22 of 23 characterized harpins, N-termini contain predicted α-helical motifs that potentially determine protein-protein interactions20 and also direct type III translocators to eukaryotic PMs. N-termini of Hpa1 and several other harpins have been shown to determine their bioactivities and recognition by plant PM-integral proteins20,21,19. In Arabidopsis, Hpa1 can localize to the outer surface of PM while it activates cellular signaling pathways15. Therefore, plant PMs must contain receptors that perceive the PM-anchored Hpa1 signal and transmit it to the cognate cellular pathways. In agreement with this hypothesis, recently we disclosed that Hpa1 expressed in yeast directly interacted with aquaporin (AQP) OsPIP1;3 from rice2,21,23,24. AQPs are intramolecular channels essential for movements of H2O, CO2, and other small substrates across biomembranes22,23. By this role, AQPs can modulate CO2 uptake and assimilation (photosynthesis) in plants22,24 and regulate water relations and many other physiological processes in all living organisms22,25–27. In plants, AQPs fall into five major phylogenetic families including the PIP family28. In most plant species, the PIP family comprises 13 members assigned to highly conserved PIP1 and PIP2 subfamilies, which consist of five (PIP1;1–PIP1;5) and eight (PIP2;1–PIP2;8) isoforms, respectively29,29. These proteins are believed to mediate transport of different substrates across plant PMs29–31. To date, however, only a small number of PIPs have been characterized in regard to their primary substrates and basic functions in most plants16,17,32,33. For example, Arabidopsis AtPIP1;2 facilitates CO2 transport in leaves32,17 and is also involved in root water relations16, AtPIP1;2, AtPIP2;1, and AtPIP2;6 coregulate rosette water transport33. These findings suggest overlapping and conserved functions of PIPs in substrate selectivity. As PMs directly face environment, PIPs are also implicated in cellular responses to a variety of extracellular signals in addition to substrate transport21,29,32,35. This functional flexibility potentially enables certain PIP isoforms to sense microbial patterns like harpins23,24.

We have explored plant sensing of Hpa1 and associated cellular pathways that regulate the bacterial virulence on rice (host plant of Xoo) and regulate both growth enhancement and immune responses of Arabidopsis (nonhost)5,14,15,35. This study is focused on Hpa1 sensing linked to the growth-enhancing effect in Arabidopsis. We show that AtPIP1;4 is a PM sensor of Hpa1 with a dual role in CO2 and H2O transport across the PM. It is technically infeasible to dissect proportions of AtPIP1;4-mediated transport of both substrates in contribution to Hpa1-induced plant growth enhancement. Instead, we present evidence that AtPIP1;4 increases its role in CO2 transport upon interacting with Hpa1, resulting in higher A\text{N} and better growth of the plant compared to the normal process without Hpa1-AtPIP1;4 interaction.

**Results**

**Hpa1 directly interacts with AtPIP1;4 at PMs of Arabidopsis cells.** We looked for Hpa1-interacting proteins in Arabidopsis by yeast two-hybrid (Y2H) systems. As a first step, a cDNA prey library from the Arabidopsis ecotype Col-0 was screened with the bait vector containing Hpa1 or Hpa1\text{ANT}. Screening of yeast transformants identified seven Hpa1-interacting clones; five of them also interacted with Hpa1\text{ANT} (Supplementary Fig. 1). The clone containing a partial sequence fragment of the AtPIP1;4 cDNA was further studied as AtPIP1;4 is a candidate that might interact with Hpa1 at the PM. The full-length coding sequence of AtPIP1;4 was isolated from Col-0 and retested by Y2H in crosswise combinations with Hpa1 or Hpa1\text{ANT} as mutual bait and preys. This crosswise assay indicated AtPIP1;4 interaction with both Hpa1 and Hpa1\text{ANT} (Supplementary Fig. 2). Proteins were further tested in a split-ubiquitin-based (SUB) Y2H system. An interaction was observed between AtPIP1;4 and Hpa1, but not between AtPIP1;4 and Hpa1\text{ANT} (Fig. 1a; Supplementary Fig. 3). Then, Hpa1 and Hpa1\text{ANT} were fused to histidine (His) and glutathione S-transferase (GST) tags15, and fusion proteins were analyzed by the in vitro pulldown assay. This assay detected AtPIP1;4 interaction with Hpa1 but not with Hpa1\text{ANT} (Fig. 1b).

To locate Hpa1-AtPIP1;4 interaction in the plant cell, we carried out bimolecular fluorescence complementation (BiFC) assays with yellow-fluorescent protein (YFP). Hpa1 and Hpa1\text{ANT} were fused to YFP N-terminal half (YFP\text{N}), generating the Hpa1-YFP\text{N} and Hpa1\text{ANT}-YFP\text{N} fusion proteins, respectively.
Meanwhile, AtPIP1;4 was fused to YFP C-terminal half (YFPC), forming the AtPIP1;4-YFPC fusion protein. An interaction was observed between AtPIP1;4-YFPC and Hpa1-YFPN, and the interaction was found at PMs of protoplasts (Fig. 1c; Supplementary Fig. 4) and leaf epidermal cells (Fig. 1d,e). The PM-localized Hpa1-AtPIP1;4 interaction was specific as interaction was absent in all negative controls, and it was also not present between Hpa1∆NT and AtPIP1;4 (Fig. 1c–e; Supplementary Fig. 4). Red-fluorescent PM probe FM4-64 was well colocalized with the YFP signal from Hpa1-AtPIP1;4 interaction, but colocalization was not observed in controls or between AtPIP1;4-YFPC and Hpa1∆NT-YFPN (Fig. 1c–e). Clearly, Hpa1 and AtPIP1;4 directly interact at the PM with the requirement for the N-terminal region of Hpa1 sequence.
AtPIP1;4 contributes to plant growth and the promoting effect of Hpa1. For use in studies to characterize physiological consequence of Hpa1-AtPIP1;4 interaction, we isolated homozygous lines of Arabidopsis T-DNA insertion mutants atpip1;4-1, atpip1;4-2, and atpip1;4-3 (Supplementary Fig. 5a). We confirmed T-DNA-indexed coding sequence of the AtPIP1;4 gene (Supplementary Fig. 5b) and also verified nullification of the gene expression in mutants (Supplementary Fig. 5c). By contrast, the gene was highly expressed in leaves of the wild-type (WT) plant irrespectively of treatment with water or an aqueous solution of Hpa1 or Hpa1ΔNT (Supplementary Fig. 5c). This suggests that insertional mutations at the coding sequence of AtPIP1;4 do not affect its responsiveness to Hpa1, or Hpa1 does not have a transcriptional effect on the gene. However, AtPIP1;4 mutations caused significant (P < 0.01) suppressions on plant growth and the promoting role of Hpa1 (Fig. 2a,b).

Plant growth was observed in 60 days after stratification and in this period, plants were treated on 15 and 30 days with water (control) and aqueous solutions of purified Hpa1 and Hpa1ΔNT, respectively (Fig. 2a). Mutants were compromised in the normal growth, and they were further impaired in Hpa1-induced growth enhancement (Fig. 2a,b). Fresh weight of WT plants was significantly (P < 0.01) increased in 20 days and then kept constant increase till 40 days after the first application of Hpa1

Figure 2. AtPIP1;4 mutation impairs plant growth and the effect of Hpa1. (a) Plants photographed at the indicated times. (b) Plant growth comparison based on fresh weight per plant. Treatment time (tt) is indicated. Data shown are means ± SEMs (n = 225 plants). Asterisks indicate significant (P < 0.01) differences between the corresponding data pairs.
compared to Hpa1∆NT or water (Fig. 2b). The effect was not found in atpip1;4 mutants; instead, Hpa1-treated mutant grew similarly to WT plants treated with Hpa1∆NT or water (Fig. 2a,b). Therefore, AtPIP1;4 mutations reduce Arabidopsis growth and further arrest the promoting effect of Hpa1.

AtPIP1;4 is required for photosynthesis and the promoting effect of Hpa1. To elucidate whether AtPIP1;4 regulates plant growth in relation to photosynthesis, we determined AN in WT and atpip1;4 leaves. Nullified AtPIP1;4 expression (Fig. 3a; Supplementary Fig. 6) caused a significant (P < 0.01) decrease in the AN level (Fig. 3b; Supplementary Fig. 6). At saturating CO2 concentration (500 μmol/mol air) and light density (750 μmol/m2/s), namely photosynthetically active photon flux density (PPFD), multiples of AN reduction in atpip1;4-1, atpip1;4-2, and atpip1;4-3 vs. WT were 43%, 41%, and 46%, respectively. Thus, AtPIP1;4 occupies a >40% proportion of AN, suggesting that photosynthesis partially requires a functional AtPIP1;4.

As the growth (Fig. 2) and AN (Fig. 3b) were highly reduced in atpip1;4-3, this mutant was used in the genetic complementation. The mutant was complemented by transformation with a recombinant vector made of full-length cDNA of the WT AtPIP1;4 gene fused at the 5′-terminus to the gene promoter and fused at the 3′-terminus to the coding sequence of green-fluorescent protein (GFP). Complemented lines Comp:GFP:1, #2, and #3 resembled WT in AtPIP1;4 expression (Fig. 3a; Supplementary Fig. 6). Nullified AtPIP1;4 expression (Fig. 3a; Supplementary Fig. 6) caused a significant (P < 0.01) decrease in the AN level (Fig. 3b; Supplementary Fig. 6). At saturating CO2 concentration (500 μmol/mol air) and light density (750 μmol/m2/s), namely photosynthetically active photon flux density (PPFD), multiples of AN reduction in atpip1;4-1, atpip1;4-2, and atpip1;4-3 vs. WT were 43%, 41%, and 46%, respectively. Thus, AtPIP1;4 occupies a >40% proportion of AN, suggesting that photosynthesis partially requires a functional AtPIP1;4.

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AtPIP1;4 facilitates CO2 transport across PMs of plant cells. As photosynthesis is limited largely by CO2 diffusion inside leaves and its availability at the site of photosynthesis under saturated PPFD, the difference of AN in WT, atpip1;4, and Comp:GFP plants presumably arose from a reduction of CO2 transport either by leaf stomata or by mesophyll cells or both. However, AtPIP1;4 was unrelated to the substomatal CO2 concentration (Ci), and to stomatal conductance (gs) either. At saturating PPFD and CO2 concentration, levels of gs and Ci (Supplementary Figs 7 and 8) were similar in all plants, without

Figure 3. AtPIP1;4 mutation and complementation alter CO2 transport and plant photosynthesis and growth. (a) Northern blotting analysis using Actin2 as a reference. (b) AN quantification based on gas exchange and gms estimate based on gas exchange and chlorophyll fluorescence. Values are means ± SEMs (n = 18 leaves). (c) Protoplast imaging (scale bar = 10 μm) and immunoblotting of leaf PM fractions using H+-ATPase as a PM-localized protein reference. (d) Plant weight (means ± SEMs; n = 54 plants). (b–d) Different letters on error bars indicate significant (P < 0.01) differences.
evident effects introduced by AtPIP1;4 mutation or complementation. Based on $A_N$–$C_i$ curve patterns (Fig. 4), $A_N$ values were similar in all plants responding to low $C_i$ (<200 μmol/mol air). By contrast, $A_N$ responses to increasing $C_i$ quantities were reduced significantly ($P < 0.01$) in mutants compared to WT or Comp:GFP plants (Fig. 4). Therefore, the role of AtPIP1;4 in $A_N$ is likely to associate with its effect on $g_m$, which limits transport of the intercellular CO2 into the cell cytosol 38.

To test this hypothesis, we used three methods to measure $g_m$ in atpip1;4 mutants compared to WT or Comp:GFP plants. The $A_N$–$C_i$ curve-fitting method revealed that $g_m$ values were ~41%, ~45%, and ~47% smaller accordingly in atpip1;4-1, atpip1;4-2, and atpip1;4-3, than in the WT plant (Supplementary Fig. 9). Coupled gas exchange and chlorophyll fluorescence analyses indicated that $g_m$ values were ~41%, ~40%, and ~43% reduced in the corresponding mutants (Fig. 3b). Based on gas exchange and stable carbon isotope 13C discrimination, the $g_m$ value was 45% smaller in the atpip1;4-3 mutant than in the WT plant (Supplementary Table 1). Hence, the $g_m$ values estimated by the three methods were consistent with each other. In essence, the AtPIP1;4-dependent $g_m$ and $A_N$ positively impacted plant growth as the growth of atpip1;4 mutants was impaired but Comp:GFP lines grew well as did WT (Fig. 3d). These analyses suggest that AtPIP1;4 indeed is a PM facilitator for CO2 transport with physiological relevance to photosynthesis and growth of the plant.

To confirm the roles of AtPIP1;4 in $g_m$ and $A_N$, we determined both parameters in WT Arabidopsis plants transformed with the AtPIP1;4:GFP fusion gene and displayed AtPIP1;4 overexpression (1;4OE) and GFP expression in the fusion form under direction by a constitutive promoter. Five 1;4OE:GFP lines were screened initially based on increased growth extents in comparison with the WT plant, and 1;4OE:GFP#1 acquired the best growth phenotype (Supplementary Fig. 10). Compared to the steady-state level of AtPIP1;4 expression in the WT plant, the AtPIP1;4:GFP fusion gene was highly expressed in 1;4OE:GFP#1 (Fig. 5a Northern blotting). In 1;4OE:GFP#1, the AtPIP1;4:GFP fusion protein was produced at a substantial amount (Fig. 3c Western blotting). AtTTG2 overexpression resulted in significant ($P < 0.01$) enhancements in growth, $g_m$ and $A_N$ (Fig. 5b,c; Supplementary Fig. 10). Accordingly, the promoting effects of Hpa1 on $g_m$, $A_N$, and growth were increased by greater extents in 1;4OE:GFP#1 than in WT (Fig. 5b,c). Therefore, the regulatory roles of AtPIP1;4 in $g_m$ and $A_N$ provide a physiological basis for Hpa1 to enhance Arabidopsis growth.

**AtPIP1;4 functions in H2O transport.** In addition to mediating mesophyll CO2 conductance, AtPIP1;4 also facilitates H2O transport across PMs of living cells. We found that de novo expression of AtPIP1;4 was able to increase osmotic water permeability ($P_i$) of African clawed frog *Xenopus laevis* oocytes. Values of $P_i$ were determined to be 22.35 ± 2.85 and 17.33 ± 2.85 μm/s in oocytes following injection with cRNAs of AtPIP1;4:His and His used as a control, respectively. The difference in $P_i$ values between AtPIP1;4:His and His were statistically significant ($P < 0.01$). This result was in agreement with
cell pressure probe measurements performed on intact plants of Arabidopsis. In cell pressure probing assays, \( \text{atpip1;4-3} \) and WT plants displayed significant differences \((P < 0.05)\) between each other in parameters of water relations except for cell volume and cell surface area (Supplementary Table 2). In particular, root cortical cell hydraulic conductivity \((L_{p rc})\) and leaf cell hydraulic conductivity \((L_{p lc})\) were higher in WT than in \( \text{atpip1;4-3} \), with a significant difference \((P < 0.05)\) in \( L_{p lc} \) (Fig. 6). Based on the differences between WT and \( \text{atpip1;4-3} \) plants, \( \text{AtPIP1;4} \) contributed to 16% (0.72 vs. 0.62) of \( L_{p rc} \) and 37% (1.67 vs. 1.22) of \( L_{p lc} \) (Supplementary Table 2). Evidently, \( \text{AtPIP1;4} \) plays a role in \( \text{H}_{2}\text{O} \) transport across PMs of Arabidopsis cells.

\( \text{AtPIP1;4} \) increases the \( \text{CO}_2 \) transport role upon interacting with \( \text{Hpa1} \). We sought to elucidate the physiological consequence of \( \text{Hpa1-AtPIP1;4} \) interaction based on the primary role of \( \text{AtPIP1;4} \) in substrate transport and the effect of \( \text{Hpa1} \). We found that the external application of \( \text{Hpa1} \) resulted in increases of \( L_{p rc} \) and \( L_{p lc} \) in the WT plant, but not in \( \text{atpip1;4-3} \) mutant (Fig. 6). Based on the differences between WT and \( \text{atpip1;4-3} \) plants, \( \text{AtPIP1;4} \) contributed to 16% (0.72 vs. 0.62) of \( L_{p rc} \) and 37% (1.67 vs. 1.22) of \( L_{p lc} \) (Supplementary Table 2). Evidently, \( \text{AtPIP1;4} \) plays a role in \( \text{H}_{2}\text{O} \) transport across PMs of Arabidopsis cells.

**Figure 5.** \( \text{AtPIP1;4} \) overexpression enhances its physiological role and the effect of \( \text{Hpa1} \). (a) Northern blotting analysis using \( \text{Actin2} \) as a reference gene and PM protein immunoblotting with antibodies specific to the indicated proteins. (b,c) Fifteen-day-old plants were treated with the indicated compounds. Twenty days later, plants were photographed; fresh weight was scored (means ± SEMs; \( n = 54 \) plants); \( g_{\text{m}} \) was estimated (means ± SEMs; \( n = 18 \) leaves) based on gas exchange and chlorophyll fluorescence; and \( A_{\text{N}} \) was determined (means ± SEMs; \( n = 18 \) leaves) based on gas exchange. Different letters on bar graphs indicate significant \((P < 0.01)\) differences.
(P < 0.01) smaller values of $g_m$ and $A_N$ in mutants (Fig. 7b; Supplementary Fig. 11). BiFC imaging showed the PM-localized interaction only between Hpa1-YFP$^N$ and AtPIP1;4-YFP$^C$ in all plants (Fig. 7c). In all plants, moreover, $g_m$ and $A_N$ were elevated significantly (P < 0.01) by cotransformation with AtPIP1;4-YFP$^C$ and Hpa1-YFP$^N$, but not with Hpa1ΔNT-YFP$^N$ and AtPIP1;4-YFP$^C$ or YFP$^N$ and YFP$^C$ (Fig. 6b; Supplementary Fig. 11). Evidently, AtPIP1;4 increases its physiological role for CO2 transport upon interacting with Hpa1 at PMs of transfected leaves.

To verify this result, we determined whether the externally applied Hpa1 was able to interact with AtPIP1;4 and affect $g_m$ and $A_N$. We treated 15-day-old plants by spraying over plant tops with water and aqueous solutions of His-Hpa1 and His-Hpa1ΔNT, respectively (Fig. 8a), isolated leaf PM proteins, and analyzed them by immunoblotting in which H$^+$-ATPase was use as a PM marker (Fig. 8b). In water or

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**Figure 6.** Arabidopsis cell hydraulic conductivity. Data shown are means ± SEMs (n = 20 cells). Different letters on error bars indicate significant (P < 0.05) differences.

**Figure 7.** AtPIP1;4 increases its physiological role by binding de novo expressed Hpa1. (a) Northern blotting and real-time RT-PCR analyses of AtPIP1;4 with the reference gene Actin2. The transcript ratio is shown as mean ± SEM (n = 6 experimental repeats). (b) Means ± SEMs (n = 18 leaves) of $g_m$ estimate based on gas exchange and $A_N$ determined by gas exchange and chlorophyll fluorescence at six hours after leaf transinfection with the indicated proteins. Different letters on bar graphs indicate significant (P < 0.01) differences. (c) YFP BiFC signals visualized on 60 hours after transinfection. FM 4–64 was utilized to mark cell outlines in red. Scale bar = 10 μm.
His-Hpa1∆NT treatment, none of PM proteins in the blot was able to hybridize with the antibody specific to GFP or His. In His-Hpa1 treatment, the AtPIP1;4-GFP fusion protein present in Comp:GFP#1 PM fraction was detected by hybridization with the GFP antibody. Meanwhile, probing with His antibody detected the His-Hpa1 fusion protein from leaf PM fractions of both WT and Comp:GFP#1 plants treated with His-Hpa1 in contrast to His-Hpa1∆NT or water. By contrast, both antibodies were not hybridized with PM protein samples from atpip1;4-3 (Fig. 8b). Furthermore, co-immunoprecipitation (Co-IP) assays revealed AtPIP1;4-GFP interaction with His-Hpa1, but not with His-Hpa1∆NT, at Comp:GFP#1 PMs (Fig. 8c). These analyses suggest that Hpa1 existed together with AtPIP1;4 or both proteins directly interacted at PMs of WT or Comp:GFP#1 plants, but not atpip1;4 mutant. This mutant grew similarly in different treatments but WT and Comp:GFP#1 growth was enhanced by His-Hpa1 treatment (Fig. 8a–d). In WT and Comp:GFP#1 plants, His-Hpa1 treatment significantly (P < 0.01) elevated levels of \( g_m \) and \( A_N \), whereas, both parameters changed little in atpip1;4-3 irrespectively of treatments (Fig. 8d). Clearly, interacting with Hpa1 enables AtPIP1;4 to boost its physiological role in CO₂ transport and further promote photosynthesis and growth of the plant.

**Discussion**

One-domain harpins are the jack of all bacterial proteins secreted by the type III secretion system, with the critical effects on bacterial virulence to host plants and both growth and immunity enhancements of nonhosts in a pathogen-independent manner\(^1,2\). With the attempt to disclose plant sensing of one-domain harpin Hpa1 and the physiological consequence, we have studied the molecular basis of Hpa1-induced Arabidopsis growth with three major results. Firstly, AtPIP1;4 is an Hpa1-interacting protein at Arabidopsis PMs (Fig. 1; Supplementary Figs S1–S4) and also a significant regulator for normal growth and Hpa1-induced growth enhancement of the plant (Fig. 2; Supplementary Fig. 10). Secondly, AtPIP1;4 plays a dual role in facilitating CO₂ and H₂O transport across the plant PM, occupying at least 40% of mesophyll conductance to CO₂ (Figs 3 and 4; Supplementary Figs 9 and 11) and up to 37% of cell hydraulic conductivity in Arabidopsis leaves (Fig. 6). Thirdly, the role of AtPIP1;4 in CO₂ transport contributes to a substantial proportion (45%) of leaf photosynthesis, and this effect is increased by AtPIP1;4 interacting with Hpa1, resulting in growth enhancement of the plant following the external
application and de novo expression of Hpa1 (Figs 5–8; Supplementary Fig. 11). These results discover the molecular mechanism that Hpa1 deploys to impact plant growth and photosynthetic physiology in a pathogen-independent manner.

To perform their physiological roles, AQPs must interact with their kinases for phosphorylation 34,40,41 and may experience additional two types of hetero-molecular interactions 35, between AQP isoforms 32–49 and between AQPs and other proteins that are neither AQPs nor kinases 50–53. Hetero-molecular interactions have been elucidated for at least four of 12 characterized members of the AQP family in mammals 44. Here, we extend this finding to plants by elucidating AtPIP1;4 interaction with Hpa1 (Fig. 1; Supplementary Figs 1–4), a one-domain harpin that relies on its N-terminal region to enhance photosynthesis and growth of Arabidopsis 52. The N-terminus of harpins contains predicted α-helix motifs that potentially determine protein-protein interactions 20, direct one-domain harpins to eukaryotic PMs 32, and may serve as a determinant of Hpa1 interaction with AtPIP1;4 (Fig. 1; Supplementary Figs 3 and 4). No matter under de novo expression (Figs 1 and 6) or external application (Fig. 7), Hpa1 is able to interact with AtPIP1;4 at the PM. A frequently questioned issue is how the externally applied harpins move across plant CMs to associate with the PMs. We ever neglect plant CW architecture and proposed that one-domain harpins have the intrinsic ability to breach plant CWs 55 and might create hole on them. Then, we supposed that harpins travel through this induced hole toward the PMs and finally bind to PM sensors, followed by cellular responses 22. In fact, this hypothesis is pointless because plant CWs are very porous and cannot block passage of large molecules, including proteins 56. Therefore, no matter how a harpin gets access to plant surfaces, it should smoothly traverse CWs and associate with PMs or interact with a PM sensor like Hpa1 interacting with AtPIP1;4. The PM-localized Hpa1-AtPIP1;4 interaction causes a physiological consequence, i.e., increasing the primary role of AtPIP1;4 in mediating mesophyll CO2 transport (Figs 3–5, 7, and 8; Supplementary Figs 9 and 11). The physiological role of AtPIP1;4 determines its function in normal growth and Hpa1-induced growth enhancement of the plant (Fig. 2).

PIPs play a direct role in H2O transport. In rosettes of Arabidopsis grown under hydroponic conditions, AtPIP1;2, AtPIP2;1, and AtPIP2;6 were highly expressed with significant contributions to water transport, whereas, AtPIP1;4 was expressed to a lower level and might be unrelated to rosette water relations 34. AtPIP1;4 was also tested but not definitely implicated in root H2O transport during lateral root growth regulated by phytohormone auxin 32. Auxin controls lateral root growth by restricting the activity of AtPIP2;1 for mediating cell hydraulic conductivity within root tissue area at the base of lateral root primordia and beneath vascular tissues. The expression of AtPIP1;4 gene in roots is induced by lateral root growth but repressed by auxin. These data are insufficient to elucidate whether AtPIP1;4 participates in auxin-regulated growth of lateral roots. In fact, the physiological role of AtPIP1;4 is not known until now.

We elucidate the physiological role of AtPIP1;4 in association with its effect on the growth and growth-promoting effect of Hpa1 in Arabidopsis. We show that AtPIP1;4 plays a dual role in H2O and CO2 transport across PMs of the plant. Based on A\textsubscript{N}-C\textsubscript{4} curve-fitting, gas exchange plus chlorophyll fluorescence, and 13\textsubscript{C} discrimination analyses, which are well accepted methods in the study of photosynthetic physiology 37,60,61, AtPIP1;4 contributes to more than 40% of mesophyll CO\textsubscript{2} conductance (Figs 3 and 4; Supplementary Figs 9 and 11). Cell pressure probe measurements suggest that AtPIP1;4 is responsible for 16% and 37% of hydraulic conductivity in roots and leaves, respectively (Fig. 6). Genetic and biochemical analyses show that AtPIP1;4 increases its CO\textsubscript{2} transport role upon interacting with Hpa1 at plant PMs, resulting in increased photosynthesis rates and enhanced growth of plants compared to the normal process without Hpa1-AtPIP1;4 interaction (Figs 7 and 8; Supplementary Fig. 11). In addition, the H\textsubscript{2}O transport role of AtPIP1;4 may also contribute to Hpa1-induced plant growth enhancement as this role increases in plants treated with Hpa1 (Fig. 6). At present, however, we don't have evidence to support this hypothesis.

In contrast to the widely accepted theory of H\textsubscript{2}O transport role as initially assigned to AQPs, PIP-facilitated CO\textsubscript{2} transport has been under debate 62–65 whilst the physiological role of individual AQP isoforms is also a matter of controversy 28,66. Recent studies support the significance of PIP1s in CO\textsubscript{2} transport 17,57,67,68 and in Arabidopsis, both AtPIP1;2,17,67 and AtPIP1;4 (Figs 3–5) have been characterized as physiologically relevant facilitators of CO\textsubscript{2} transport across PMs. However, none of the PIP1s play a full role in mediating CO\textsubscript{2} transport. Instead, mutants or gene-silenced plants are still able to assimilate CO\textsubscript{2} without chlorosis. Presumably, additional PIPs or other channels also govern transport of CO\textsubscript{2} across PMs, and they may function as a consortium to implement a full function in the process 24. This claim agrees with recent findings on the functional specificity and redundancy of PIPs. For example, AtPIP1;2, AtPIP2;1, and AtPIP2;6 share their functions in leaf water relations 32,34. AtPIP2;1 also regulates cell hydraulic conductivity in roots 32, and AtPIP1;4 functions in both roots and leaves to regulate hydraulic conductivity (Fig. 6). As one more example, tobacco Nicotiana tabacum NtAQP1 is unrelated to the shoot H\textsubscript{2}O transport; instead, it increases gs and A\textsubscript{N}, resulting in enhanced plant growth 40. In addition, the predicted NtAQP1 protein (accession number CAA04750) does not share similarities with any Arabidopsis AQPs, except for an 88% identity in two short regions (2/3–181/182 and
183/182–287/286) to a hypothetical protein (accession number EFH49212.1). Hence, AQPs are highly diverse with overlapped and redundant functions in plants\(^{21,28}\), explaining the dual role of AtPIP1;4 in CO\(_2\) and H\(_2\)O transport.

In summary, our data offer robust evidence for the molecular mechanism by which one-domain harpin Hpa1 interacts with AtPIP1;4 to facilitate CO\(_2\) transport in Arabidopsis. This finding should stimulate further studies to explore the structural basis of AQP-partnering protein interactions\(^{35}\). AQPs possess six \(\alpha\)-helical TM (TM1–TM6) domains that are tilted along the plane of PM and linked one to the other by five connecting loops (LA–LE)\(^{21,28}\), LB, LD, and both N-terminal and C-terminal regions locate inside the cell and potentially bind to cytosolic substrates\(^{31,69}\). Inversely, LA, LC, and LE face the apoplasm and have the opportunity to contact with apoplastic substrates\(^{70}\). Presumably, LA, LC, and LE enable PIPs to sense biotic signals and therefore extend their functional scopes beyond substrate transport\(^{42,71}\). This structural feature and functional flexibility of AQPs provide the molecular basis of AtPIP1;4 interaction with Hpa1 and the subsequent effect on photosynthesis. Studies in the future to characterize whether the topological distribution of AtPIP1;4 on the PM alters upon interacting with Hpa1 will be critical to elucidate mechanisms that underpin Hpa1-AtPIP1;4 interaction and the physiological consequence.

**Methods**

**Plant material and growth conditions.** Seeds of Arabidopsis ecotypes Col-0 and Col-3 (stock numbers CS28166 and CS28171) and Col-3 mutants attip1;4-1, attip1;4-2, and attip1;4-3 (CS879846, CS872202, and CS870828) were purchased from The Arabidopsis Information Resource (TAIR, www.arabidopsis.org). Homozygous mutants were isolated, transgenic plants generated, and all plant seeds maintained in H.D. lab. Seeds were germinated in flat plastic trays filled with a substrate containing peat, sand, and vermiculite (1:1:1 v/v). Three days later, germinal seedlings were moved into 60-ml pots (3 plants per pot) filled with the same substrate. Seeds were incubated and plants were grown in plant growth chambers under 24 ± 1°C and 12-hour light at 250 ± 50 μmol quanta/m\(^2\)/sec.

**Protein interaction assays.** Y2H system III (Clontech) was used in screening of cDNA prey library CD4-22 from Arabidopsis ecotype Col-0 (TAIR) with a bait vector containing the hpa1 or hpa1ΔNT gene\(^{15}\). Positive clones were sequenced and retested in the system. The positive clone containing a partial sequence fragment of AtPIP1;4 was further tested in pairwise combination with Hpa1 or Hpa1ΔNT as mutual bait and preys. Full-length AtPIP1;4 cDNA was obtained by RT-PCR with mRNA isolated from leaves of Arabidopsis ecotypes Col-0 and Col-3. Sequences of both RT-PCR products were confirmed to be 100% identical with each other and with the published sequences. AtPIP1;4 in combination with Hpa1 or Hpa1ΔNT was tested in SUB Y2H system (Dualsystems). This system was also employed to test protein combinations between Hpa1 as bait and each of OsPIP1;1–1;3 isoforms as a prey. To carry out in vitro pulldown assays, AtPIP1;4 was linked to a His\(_{10}\) tag while Hpa1 or Hpa1ΔNT was fused to both His\(_{10}\) and GST tags\(^{8,15}\). The AtPIP1;4-His fusion protein was produced in *Pichia pastoris*\(^{74}\) while GST-His-Hpa1 and GST-His-Hpa1ΔNT were produced in *Escherichia coli*\(^{1}\). Proteins were purified by nickel chromatography\(^4\) and used in pulldown assays\(^{74}\). Co-IP was performed on leaf PM fraction by using the Pierce\® Co-IP Kit (Thermo Sci.) as per the manufacturer’s instruction. For in vivo molecular interaction analyses and other tests, 35-day-old plants and their fourth leaves were used unless specified elsewhere. YFP BiFC tests were conducted on leaves and leaf protoplasts\(^{75,76}\). Cell outlines were visualized by a 5 μg/ml aqueous solution of PM marker FM4-64 (Invitrogen), which was applied by immersing protoplasts or leaves in tubers on ice within two minutes before confocal microscopy. The FM4-64 signal was captured with 734-nm emission and 558-nm excitation while the YFP signal was captured as out.

**Mutant screening.** T-DNA-insertional Arabidopsis mutants attip1;4-1, attip2;4-2, and attip1;4-3 were generated previously by transformation of Col-3 with plant binary vector pDAP101 (TAIR). This vector carries the Basta-resistant gene (*Basta*) as a selective marker. Basta is a commercial brand name of the herbicide N-phosphonomethyl glyline, namely glyphosate. A commercial supply of Basta as a 10% aqueous solution of PM marker FM4-64 (Invitrogen), which was applied by immersing protoplasts or leaves in tubers on ice within two minutes before confocal microscopy. The FM4-64 signal was captured with 734-nm emission and 558-nm excitation while the YFP signal was captured as previously described\(^{75}\).
integrated into the plant genome. If all of 10 plant individuals in T4 generation derived from a single stock of T3 seeds were resistant to Basta and contained the T-DNA insert and Basta gene at correct sites, seeds in the stock were regarded as homozygous at the mutation locus and their progenies were used subsequently in all of the experiments.

Genetic complementation. The genetic complementation unit was constructed with the plant binary pCAMBIA1301 vector (CAMBIA), which contains a GFP gene and the cauliflower mosaic virus the cauliflower mosaic virus 35S promoter (P35S). The promoter region and coding sequence of AtPIP1;4 were cloned from Col-3 DNA and RNA by PCR and RT-PCR, respectively. Their sequences were confirmed by sequencing with clones in the pMD19-T vector (Takara). Confirmed AtPIP1;4 promoter and cDNA sequences were inserted into pCAMBIA1301 at the front of P35S and the right border to form the genetic complementation union and exclude P35S in the recombinant vector. The recombinant vector was transferred into cells of the Agrobacterium tumefaciens strain EHA105, and a suspension of recombinant EHA105 cells was used in transformation of atpip1;4-3 through blossom infiltration77. Transgenic plants were screened and characterized as previously described77,78 and T3 homozygous progenies were used in this study.

Gene overexpression. The AtPIP1;4-GFP fusion gene was inserted into the plant binary vector pCAMBIA1301 between the 3′-terminal end of P35S and front of the right border77. The recombinant vector was transferred into the genome of WT Col-3 plants under mediation by A. tumefaciens13. Transgenic plants were created, screened, and characterized by conventional protocols12,77. T3 homozygous progenies were used in this study. Gene overexpression was verified by real-time RT-PCR and Northern blotting analyses, and production of the AtPIP1;4-GFP fusion protein was detected by immunoblotting with specific GFP antibody (Novagen) or His antibody (Merk). In immunoblotting analyses, PM marker protein H^+ATPase was as a reference and probed with the specific antibody (Santa Cruz).

Plant treatment and growth scoring. Prokaryotic expression vectors used for production of Hpa1-His and Hpa1ΔNT-His fusion proteins were constructed previously15. Proteins were produced in E. coli, purified by nickel chromatography, and treated with an enterokinase to remove His4. Purified proteins were prepared as aqueous solution stocks and their concentrations were determined4. Based on known effective dosage of harpins4,15,16,78, every protein was used at a final concentration of 10μg/ml in an a water-diluted solution and applied by the aid of an atomizer to spray over tops of plants on 15 and 30 days after stratification. Plants were treated similarly with pure water in control. Plant growth extents were quantified as fresh weight at time intervals after treatment.

Gene expression analysis. Information on genes tested in this study is provided in Supplementary Table 3. Previously described methods4 were used in Northern blotting, RT-PCR, and real-time RT-PCR analyses for AtPIP1;4 expression. The constitutively expressed EF1α and Actin2 genes were used as references. In real-time RT-PCR, cDNA templates were analyzed together with temple-absent controls. Relative level of AtPIP1;4 expression was quantified as the transcript quantity ratio of AtPIP1;4 to a reference gene.

Immunoblotting. Leaf and cytoplasmic PM proteins were isolated13 and analyzed by immunoblotting79. Protein blots were incubated with every of the specific antibodies and hybridized to horseradish peroxidase-conjugated goat antimouse immunoglobulin G from the BeyoECL Plus kit (Beyotime).

Gas exchange measurements. Plants used in gas exchange measurements were grown in substrate-overfilled pots61 for 35 days and measurements were performed on top third and fourth fully unfolded leaves unless specified elsewhere. Leaf gas exchange was measured with the Li-6400XT portable photosynthesis system and the equipped 2-cm² leaf chamber (Li-Cor. Biosci.). Detailed measurements on single leaves were performed by following the manufacturer’s instructions and previously described experimental procedures61. During measurements, relative humidity in leaf chamber was constantly maintained at 45% and leaf temperature was kept at 25°C. CO₂ concentrations at the inlet and outlet of the leaf chamber were monitored by the non-dispersive infrared gas analyzer installed in the system. PPFD was controlled by adjusting intensities of the lamp-house irradiation. Readings of A_N, C_i and more related photosynthetic parameters were documented automatically by the S-501 digital monitor integrated into the Li-6400XT system. For every plant genotype or treatment, instantaneous gas exchange measurements on a single leaf were performed every two hours in the light cycle of plant growth to obtain sufficient and reliable data, which were used subsequently in estimation of g_m by curve-fitting, chlorophyll fluorescence quenching, and 13C discrimination analyses80,81.

g_m estimate by the A_N-C_i curve-fitting method. Assessments of g_m by the curve-fitting method require a large number of data points to be reliable82,83, it was only possible to perform this estimation in young leaves77,61. Therefore, photosynthesis responses to changes in light intensity and CO₂ concentration were analyzed through gas exchange measurements on top 3–5 expanded leaves of 35-day-old plants. A_N–C_i curves were established by measuring A_N at PPFD 750μmol/m²/sec and a range of C_i
(1000–0.01 mol/mol air on a 100-descendent gradient). The value of $g_m$ was estimated by the $A_N$–$C_i$ curve-fitting method.

**$g_m$ estimate based on gas exchange and chlorophyll fluorescence.** Gas exchange measurements coupled with chloroplast fluorescence monitoring were conducted on 35-day-old plants. Gas exchange was measured under saturating light (PPFD 750 μmol/m²/sec) and CO₂ concentration of 500 μmol/mol air. The respiration rate in light ($R_L$) and CO₂ photocompensation point in the absence of $R_N$ ($P_N^*$) were estimated by a previously described method. Then, both parameters were used in $g_m$ assessments together with the photosynthetic electron transport rate ($J$) obtained from the chlorophyll fluorescence quenching analysis.

Chlorophyll fluorescence was analyzed with the Multiple Excitation Wavelength Chlorophyll Fluorescence Analyzer (Heinz Walz GmbH). This analyzer was operated under conditions of the leaf temperature kept at 25°C, the chamber CO₂ concentration adjusted to 500 μmol/mol air, and the leaf-to-air vapor pressure deficit maintained at 1.2 kPa. Leaves were first adapted to dark for 20 minutes and then adjusted to PPFD 1500 μmol/m²/sec, and chlorophyll fluorescence was measured on three 3-mm² circular bars located between leaf nervures in a single leaf. The value of $J$ from the fluorescence analyzer ($J_{flu}$) and the photosynthetic parameters $A_N$, $R_L$, and $P_N^*$ obtained from gas exchange measurements were utilized to estimate $g_m$ using equation (1):

$$
g_m = A_N \div [C_i-G_\lambda \times (J_{flu} + 8 \times (A_N + R_L))] \div [J_{flu} - 4 \times (A_N + R_L)] \quad \text{(1)}
$$

**$g_m$ Estimate by $^{13}$C Discrimination in Recently Synthesized Carbohydrates.** Because $g_m$ assessments based on gas exchange in leaves and stable carbon isotope discrimination in recently synthesized carbohydrates require a large amount of pulverized and lyophilized leaf material (100 mg per sample), the experiments were conducted with large-scaled pools of plant populations. For every Arabidopsis genotype, therefore, 300 plants in 100 pots were grown for 35 days under the same conditions. Totally 210 plants of a genotype were chosen based on the criterion of uniform growth and used as a material pool for gas exchange and $^{13}$C discrimination analyses. Immediately after gas exchange measurements performed on top 3–5 expanded leaves of 10 plant individuals randomly selected from the material pool, equivalent leaves were excised from all of the 210 plants and pulverized thoroughly with liquid nitrogen. Resulting leaf powders were lyophilized, weighed, stored at −65°C when necessary, and processed (directly or after thaw) to prepare soluble sugars, which were subsequently used for $^{13}$C discrimination ($\Delta$) in recently synthesized carbohydrates as previously described.

Soluble sugars were extracted from low-molecular weight compounds (LMWC), which was isolated from lyophilized leaf powders and purified by ion-exchange chromatography. A critical step in LMWC purification was ion exchange chromatography with the cation-exchange resin DOWEX 50W and the anion-exchange resin DOWEX 1. The former resin was used for the separation of amino acids from organic acids and sugars, and the latter was used to separate organic acids from soluble sugars. Concentrations of $^{13}$C in purified sugar preparations and in source air as well were determined by coupled analysis with an element analyzer (EA) and an isotope ratio mass spectrometer (IRMS). The IRMS facility used in this analysis is a Finnigan MAT DeltaPlus XP isotope ratio mass spectrometer (Thermo Finnigan MAT), which was coupled to a Flash EA 1112 Series elemental analyzer (Thermo Italy) through a six-port valve and a universal interface for EA-IRMS coupling (ConFlo III, Thermo Finnigan). With readings from the Flash EA analyzer, the observed $\Delta$ ($\Delta_{obs}$) was calculated using equation (2):

$$\Delta_{obs} = \frac{\delta_a - \delta_p}{1 + \delta_p} \quad \text{(2)}$$

Here, $\delta_a$ and $\delta_p$ are the isotope compositions of source air and plant material, respectively, relative to international standard Vienna-Pee Dee Belemnite.

Values of $g_m$ were determined by comparing $\Delta_{obs}$ with predicted discrimination ($\Delta_e$) as follows: $a$ (empirical proxy 4.4‰) was the fractionation during diffusion in air; $b$ (28.2%) was the discrimination associated to carboxylation reactions; $c_i$ was obtained from the gas exchange measurement; and $C_i$ was the air CO₂ concentration adjusted during the measurement. Then, $\Delta_{e}$ was calculated using equation (3):

$$\Delta_{e} = a + (b-a) \times C_i \div C_a \quad \text{(3)}$$

In addition to $\Delta$, discrimination parameters also required for estimation of $g_m$ were empirical values of the fractionation during the dissolution of CO₂ ($c_e$; 1.1‰) and the discrimination by CO₂ diffusion in the liquid phase ($a_i$; proxy 0.7‰) and by photorespiration ($f$; 8%). These discrimination parameters were used along with gas exchange parameters $P$*, the dark respiration rate ($R_0$), and the carboxylation efficiency ($k$) to calculate $g_m$ according to equation (4):

$$g_m = \{(b-e_e-a_i) \times A_N \div C_a\} \div \{(\Delta_e-\Delta_a) \div [(e \times R_0 \div k + f \times P^*) \div C_a] \}
\quad \text{(4)}$$
Values of $g_m$ from this calculation and estimated by the $A_{co2}$ curve-fitting method were compared to evaluate the reliability of $g_m$ assessments based on the gas exchange and isotope discrimination protocol.

**Oocyte Pf determination.** For expression in *X. laevis* oocytes, capped cRNA of PIP1:4:His or His was synthesized in vitro from Not I-linearized pGH19 plasmid and purified with the RNeasy Mini Kit (Qiagen). Stage IV–V oocytes were defolliculated and injected with 5ng of cRNA or 50 nl of diethyl pyrocarbonate-treated water in control. Injected oocytes were incubated for 2–3 days at 18°C in ND96 culture medium. $P_f$ was estimated by the oocyte swelling assay. Oocytes were transferred into liquid ND96 medium diluted to 50 milliosmolar with distilled water, and the time course of volume increase was monitored at room temperature by videomicroscopy with an on-line computer.

**Measurements of cell hydraulic parameters.** $Lp_c$ and $Lp_e$ were determined by cell pressure probe (CPP) measurements performed on root segments and leaf blades. Pulled glass microcapillaries were beveled to a tip diameter of 5–7 μm, filled with type AS4 silicon oil (Wacker), and mounted vertically on a pressure probe. For $Lp_e$ measurement, root segment was excised from plants grown in hydroponic conditions, and was placed on a metal sledge that was covered with filter paper. An aerated plant culturing solution was circulated around the root segment to maintain hydration. Cortical cells from second to fourth layer and at 5–8 cm distance from the root apex were punctured using a CPP. Cell turgor was restored by gently pushing the meniscus to a position close to the surface of the root, and the values of cell turgor pressure were recorded by a computer. The half time ($T_{1/2}$) of hydrostatic water flow across cell membrane, which is inversely proportional to cell hydraulic conductivity ($T_{1/2} \propto 1/Lp$) was obtained from pressure relaxation curves with the aid of the probe. $T_{1/2}$ measurements for a given cell were finished within 10 min after root excision. $Lp_c$ was calculated from the measured $T_{1/2}$. For $Lp_e$ measurement, a mature young leaf blade on the plant was fixed onto a metal support and leaf cells were punctured using a CPP. Upon a successful puncture, cell turgor was restored by gently moving the meniscus to a position close to the surface of the leaf. $Lp_e$ was determined as for roots.

**Statistical analysis.** All experiments were repeated at least three times with similar results. Quantitative data were analyzed with IBM SPSS19.0 software package. Homogeneity-of-variance in data was determined by Levene test, and formal distribution pattern of the data was confirmed by the Kolmogorov-Smirnov test and P-P Plots. Data were subjected to analysis of variance along with Fisher’s least significant difference test.

**References**

1. Choi, M. S., Kim, W. C. & Oh, C. S. Harpins, multifunctional proteins secreted by Gram-negative plant-pathogenic bacteria. *Mol. Plant-Microbe Interact.* **26**, 1115–1122 (2013).
2. Zhu, W., MaGhaunu, M. M. & White, F. F. Identification of two novel hrp-associated genes in the *hrp* gene cluster of *Xanthomonas oryzae* pv. *oryzicola*, that induce disease resistance and enhanced growth in rice. *Phytopathology.* **98**, 781–791 (2008).
3. Ausubel, F. M. Are innate immune signaling pathways in plants and animals conserved? *Nat. Immunol.* **6**, 973–979 (2005).
4. Torres, M. A. ROS in biotic interactions. *Physiol. Plant.* **138**, 414–429 (2010).
5. Dong, H. et al. Harpin induces disease resistance in *Arabidopsis* through the systemic acquired resistance pathway mediated by salicylic acid and the NIM1 gene. *Plant J.* **20**, 207–215 (1999).
6. Dong, H. P. et al. Downstream divergence of the ethylene signaling pathway for harpin-stimulated Arabidopsis growth and insect defense. *Plant Physiol.* **136**, 3628–3638 (2004).
7. Fu, M. Q. et al. Transgenic expression of a functional fragment of harpin protein Hpa1 in wheat induces the phloem-based defense to English grain aphid. *J. Exp. Bot.* **65**, 1349–1453 (2014).
8. Zhang, C. L. et al. Harpin-induced expression and transgenic overexpression of the phloem protein gene *AtPP2-A1* in *Arabidopsis* repress phloem feeding of the green peach aphid *Myzus persicae*. *BMC Plant Biol.* **11**, 1 (2011).
9. Sang, S. L. et al. Apoplastic and cytoplasmic location of harpin protein Hpa1$_{brca}$ plays different roles in *H$_2$O$_2* generation and pathogen resistance in *Arabidopsis*. *Plant Mol. Biol.* **79**, 375–391 (2012).
10. Li, X. J. et al. Plant growth enhancement and associated physiological responses are coregulated by ethylene and gibberellin in *Arabidopsis*. *Plant J.* **39**, 127–137 (2014).
11. Li, X. J. et al. The Hpa1 harpin needs nitroxyl terminus to promote vegetative growth and leaf photosynthesis in *Arabidopsis*. *J. Bacteriol.* **186**, 877–886 (2004).
12. Heffelft, M., Pater, D., Hanson, D. T. & Kaldenhoff, R. The *Arabidopsis thaliana* aquaporin AtIP1:2 is a physiologically relevant *CO2* transport facilitator. *Plant J.* **67**, 789–804 (2011).
13. Uehlein, N., Sperling, H., Heckwolf, M. & Kaldenhoff, R. The *Arabidopsis* aquaporin IP1:2 rules cellular *CO2* uptake. *Plant Cell Environ.* **35**, 1077–1083 (2012).
14. Ji, H. T. & Dong, H. S. Key steps in type III secretion system (T3SS) towards translocon assembly with potential sensor at plant defense. *BMC Mol. Biol.* **11**, 20 (2010).
15. Ji, H. T. & Dong, H. S. Key steps in type III secretion system (T3SS) towards translocon assembly with potential sensor at plant defense. *BMC Plant Biol.* **11**, 20 (2010).
16. Heffelft, M., Pater, D., Hanson, D. T. & Kaldenhoff, R. The *Arabidopsis thaliana* aquaporin AtIP1:2 is a physiologically relevant *CO2* transport facilitator. *Plant J.* **67**, 789–804 (2011).
17. Uehlein, N., Sperling, H., Heckwolf, M. & Kaldenhoff, R. The *Arabidopsis* aquaporin IP1:2 rules cellular *CO2* uptake. *Plant Cell Environ.* **35**, 1077–1083 (2012).
18. Ji, H. T. & Dong, H. S. Key steps in type III secretion system (T3SS) towards translocon assembly with potential sensor at plant defense. *BMC Mol. Biol.* **11**, 20 (2010).
19. Oh, C. S. & Beer, S. V. AtHIPM, an orthology of the apple HrpN-interacting protein, is a negative regulator of plant growth and mediates the growth-enhancing effect of HrpN in *Arabidopsis*. *Plant Physiol.* **145**, 426–436 (2007).
20. Gomes, D. et al. Aquaporins are multifunctional water and solute transporters highly divergent in living organisms. *Biox. Biophys. Acta.* **1788**, 1213–1228 (2009).
21. Verkman, A. S. Aquaporins. *Curr. Biol.* 23, R52–R55 (2013).
22. Katohara, M. & Hanba, Y. T. Barley plasma membrane intrinsic proteins (PIP Aquaporins) as water and CO₂ transporters. *Pflugers Arch.* 456, 687–691 (2008).
23. Ito, F. et al. CO₂ permeability of cell membranes is regulated by membrane cholesterol and protein gas channels. *FASEB J.* 26, 5182–5191 (2012).
24. Jang, H. Y., Yang, S. W., Carlson, J. E., Ko, Y. G. & Ahn, S. J. Two aquaporins of *Jatropha* are regulated differentially during drought stress and subsequent recovery. *J. Plant Physiol.* 170, 1028–1038 (2013).
25. Jeong, S. Y., Kim, J. H., Lee, W. O., Dahms, H. U. & Han, K. N. Salinity changes in the anadromous river pufferfish, *Takifugu obscurus*, mediate gene regulation. *Fish Physiol. Biochem.* 40, 205–219 (2013).
26. Xiong, Y. et al. Expression of aquaporins in human embryos and potential role of AQP3 and AQP7 in preimplantation mouse embryo development. *Cell Physiol. Biochem.* 31, 649–658 (2013).
27. Abascal, F., Iriarriz, I. & Zardoya, R. Diversity and evolution of membrane intrinsic proteins. *Biochim. Biophys. Acta.* 1840, 1468–1481 (2014).
28. Maurel, C. Plant aquaporins: novel functions and regulation properties. *FEBS Lett.* 581, 2227–2236 (2007).
29. Maurel, C., Verdoucq, L., Luu, D. T. & Santoni, V. Plant aquaporins: membrane channels with multiple integrated functions. *Annu. Rev. Plant Biol.* 59, 595–624 (2008).
30. Qin, Z. J., Song, J. J., Chaumont, F. & Ye, Q. Differential responses of plasma membrane aquaporins in mediating water transport of cucumber seedlings under osmotic and salt stresses. *Plant Cell Environ.* 38, 461–473 (2015).
31. Piot, B. et al. Auxin regulates aquaporin function to facilitate lateral root emergence. *Nat. Cell Biol.* 4, 991–998 (2012).
32. Xu, Y. et al. A banana aquaporin gene, *MapPIP1;1*, is involved in tolerance to drought and salt stresses. *BMC Plant Biol.* 14, 59 (2014).
33. Prado, K. et al. Regulation of Arabidopsis leaf hydraulics involves light-dependent phosphorylation of aquaporins in veins. *Plant Cell.* 25, 1029–1039 (2013).
34. Ji, H. T. & Dong, H. S. Biological significance and topological basis of aquaporin-partnering protein-protein interactions. *Plant Signal. Behav.* 10, in press (2015).
35. Flexas, J. et al. Mesophyll conductance to CO₂ in *Arabidopsis thaliana*. *New Phytol.* 175, 501–511 (2007).
36. Pons, T. L. et al. Estimating mesophyll conductance to CO₂: methodology, potential errors, and recommendations. *J. Exp. Bot.* 60, 2217–2234 (2009).
37. Kaldenhoff, R. Mechanisms underlying CO₂ diffusion in leaves. *Plant Cell Physiol.* 456, 481–490 (2014).
38. Hachez, C. et al. Short-term control of maize cell and root water permeability through plasma membrane aquaporin isoforms. *Plant Cell Environ.* 35, 185–198 (2012).
39. Kelly, G. et al. Relationship between hexokinase and the aquaporin PIP1 in the regulation of photosynthesis and plant growth. *PLoS One.* 9, e87888 (2014).
40. Temmei, Y. et al. Water channel activities of *Mimosa pudica* plasma membrane intrinsic proteins are regulated by direct interaction and phosphorylation. *FEBS Lett.* 579, 4417–4422 (2005).
41. Bienert, G. P. et al. A conserved cysteine residue is involved in disulfide bond formation between plant plasma membrane aquaporin monomers. *J. Exp. Bot.* 64, 101–111 (2012).
42. Hachez, C., Besserer, A., Chevalier, A. S. & Chaumont, F. Insights into plant plasma membrane aquaporin trafficking. *Trends Plant Sci.* 18, 344–352 (2013).
43. Hachez, C. et al. The Arabidopsis abiotic stress-induced TSPO-related protein reduces cell-surface expression of the aquaporin PIP2;7 through protein-protein interactions and autophagic degradation. *Plant Cell.* 26, 4974–4990 (2014).
44. Otto, B. et al. Aquaporin tetramer composition modifies the function of tobacco aquaporins. *J. Biol. Chem.* 285, 31253–31260 (2010).
45. Wudick, M. M., Luu, D. T. & Maurel, C. A look inside: localization patterns and functions of intracellular plant aquaporins. *New Phytol.* 184, 289–302 (2009).
46. Yaneff, A. et al. Heteromerization of PIP aquaporins affects their intrinsic permeability. *Proc. Natl. Acad. Sci. USA* 111, 231–236 (2014).
47. Zelazny, E. et al. FRET imaging in living maize cells reveals that plasma membrane aquaporins interact to regulate their subcellular localization. *Proc. Natl. Acad. Sci. USA* 104, 12359–12364 (2007).
48. Zelazny, E. et al. An N-terminal diacidic motif is required for the trafficking of maize aquaporins ZmPIP2;4 and ZmPIP2;5 to the plasma membrane. *Plant J.* 57, 346–355 (2009).
49. Galizia, L. et al. Functional interaction between AQP2 and TRPV4 in renal cells. *J. Cell Biochem.* 113, 580–589 (2012).
50. Hu, S., Wang, B., Qi, Y. & Lin, H. The Arg233Ile AQP0 mutation disturbs aquaporin O-calmodulin interaction causing polymorphic congenital cataract. *PLoS One.* 7, e37637 (2012).
51. Nakazawa, Y. et al. The effect of interaction between aquaporin 0 (AQP0) and the filesin tail region on AQP0 water permeability. *Mol. Vis.* 17, 3191–3199 (2011).
52. Tamma, G. et al. Integrin signaling modulates AQP2 trafficking via Arg-Gly-Asp (RGD) motif. *Cell Physiol. Biochem.* 27, 739–748 (2011).
53. Stohamm, J. & Hedfalk, K. Unraveling aquaporin interaction partners. *Biochim. Biophys. Acta.* 1800, 1614–1623 (2014).
54. Kvitko, B. H., Ramos, A. R., Morello, J. E., Oh, H. S. & Collmer, A. Identification of harpins in *Pseudomonas syringae pv. tomato* DC3000, which are functionally similar to HrpK1 in promoting translocation of type III secretion system effectors. *J. Bacteriol.* 189, 8059–8072 (2007).
55. Wang, D. F. et al. Transgenic expression of the functional fragment Hpa1α–α of the harpin protein Hpa1 imparts enhanced resistance to powdery mildew in wheat. *Plant Dis.* 98, 448–453 (2014).
56. Kim, S. G. et al. The RNase activity of rice probenazole-induced protein1 (PBZ1) plays a key role in cell death in plants. *Mol Cells.* 31, 25–31 (2011).
57. Alexandersson, E. et al. Whole gene family expression and drought stress regulation of aquaporins. *Plant Mol. Biol.* 59, 469–484 (2005).
58. Alexandersson, E. et al. Transcriptional regulation of aquaporins in accessions of Arabidopsis in response to drought stress. *Plant J.* 61, 650–660 (2010).
59. Chen, W. et al. Involvement of rose aquaporin RhPIP1;1 in ethylene-regulated petal expansion through interaction with RhPIP2;1. *Plant Mol. Biol.* 83, 219–233 (2013).
60. Flexas, J. et al. Mesophyll diffusion conductance to CO₂: an unappreciated central player in photosynthesis. *Plant Biol.* 19, 3125–3131 (2017).
64. Kaldenhoff, R. Mechanisms underlying CO₂ diffusion in leaves. *Curr. Opin. Plant Biol.* 15, 276–281 (2012).
65. Sagardoy, R. et al. Stomatal and mesophyll conductances to CO₂ are the main limitations to photosynthesis in sugar beet (*Beta vulgaris*) plants grown with excess zinc. *New Phytol.* 187, 145–158 (2010).
66. Yool, A. J. & Campbell, E. M. Structure and functional relevance of aquaporin dual water and ion channels. *Mol. Aspects Med.* 33, 553–561 (2012).
67. Heckwolf, M., Pater, D., Hanson, D. T. & Kaldenhoff, R. The *Arabidopsis thaliana* aquaporin AtPIP1;2 is a physiologically relevant CO₂ transport facilitator. *Plant J.* 67, 795–804 (2011).
68. Heinen, R. B. et al. Expression and characterization of plasma membrane aquaporins in stoma complexes of *Zea mays*. *Plant Mol. Biol.* 86, 335–350 (2014).
69. Lindsey Rose, K. M. et al. The C terminus of lens aquaporin 0 interacts with the cytoskeletal protein filensin and CP49. *Invest. Ophthalmol. Vis. Sci.* 47, 1562–1570 (2006).
70. Jin, M., Berrott, J., Chen, L. & ONeil, R. G. Hypotonicity-induced TRPV4 function in renal collecting duct cells: modulation by progressive cross-talk with Ca²⁺-activated K⁺ channels. *Cell Calcium* 51, 131–139 (2012).
71. Ruiz-Lozano, J. M. et al. Exogenous ABA accentuates the differences in root hydraulic properties between mycorrhizal and non-mycorrhizal maize plants through regulation of PIP aquaporins. *Plant Mol. Biol.* 70, 565–579 (2009).
72. Bienert, G. P. & Chaumont, F. Aquaporin-facilitated transmembrane diffusion of hydrogen peroxide. *Biochim. Biophys. Acta.* 1840, 1596–1604 (2014).
73. Hooijmaijers, C. et al. Hydrogen peroxide permeability of plasma membrane aquaporins of *Arabidopsis thaliana*. *J. Plant Res.* 125, 147–153 (2012).
74. Karlsson, M. et al. Reconstitution of water channel function of an aquaporin overexpressed and purified from *Pichia pastoris*. *FEBS Lett.* 537, 68–72 (2003).
75. Wang, Y. P. et al. *Nicotiana tabacum* TTG1 contributes to ParA1-induced signalling and cell death in leaf trichomes. *J. Cell Sci.* 122, 2673–2685 (2009).
76. Li, B. Y. et al. Tobacco TTG2 suppresses resistance to pathogens by sequestering NPR1 from the nucleus. *J. Cell Sci.* 125, 4913–4922 (2012).
77. Liu, R. X. et al. Transcription factor AtMYB44 regulates induced expression of the ETHYLENE INSENSITIVE2 gene in *Arabidopsis* responding to a harpin protein. *Plant-Microbe Interact.* 24, 377–389 (2011).
78. Dong, H. P. et al. Downstream divergence of the ethylene signaling pathway for harpin-stimulated Arabidopsis growth and insect defense. *Plant Physiol.* 136, 3628–3638 (2004).
79. Wu, C. Q. et al. Ectopic expression of the rice lumazine synthase gene contributes to defense responses in transgenic tobacco. *Phytochemistry.* 100, 573–581 (2010).
80. Sun, W., Resco, V. & Williams, D. G. Diurnal and seasonal variation in the carbon isotope composition of leaf dark-respired CO₂ in velvet mesquite (*Prosopis velutina*). *Plant Cell Environ.* 32, 1390–1400 (2009).
81. Sun, W., Resco, V. & Williams, D. G. Nocturnal and seasonal patterns of carbon isotope composition of leaf dark-respired carbon dioxide differ among dominant species in a semiarid savanna. *Oecologia* 164, 297–310 (2010).
82. Ether, G. J., Livingston, N. J., Harrison, D. L., Black, T. A. & Moran, J. A. Low stomatal and internal conductance to CO₂ versus Rubisco deactivation as determinants of the photosynthetic decline of ageing evergreen leaves. *Plant Cell Environ.* 29, 2168–2184 (2006).
83. Harley, P. C., Loreto, F., Di, Marco, G. & Sharkey, T. D. Theoretical considerations when estimating the mesophyll conductance to CO₂ flux by analysis of the response of photosynthesis to CO₂. *Plant Physiol.* 98, 1429–1436 (1992).
84. Evans, J. R., Sharkey, T. D., Berry, J. A. & Farquhar, G. D. Carbon isotope discrimination measured concurrently with gas exchange to investigate CO₂ diffusion in leaves of higher plants. *Aust. J. Plant Physiol.* 13, 281–292 (1986).
85. Wanek, W., Heintel, S. & Richter, A. Preparation of starch and other carbon fractions from higher plant leaves for stable carbon isotope analysis. *Rapid Commun. Mass Spectrom.* 15, 1136–1140 (2001).
86. Sun, W., Resco, V. & Williams, D. G. Environmental and physiological controls on the carbon isotope composition of CO₂ respired by leaves and roots of a C3 woody legume (*Prosopis velutina*) and a C4 perennial grass (*Sporobolus wrightii*). *Plant Cell Environ.* 35, 567–577 (2012).
87. Richter, A. et al. Preparation of starch and soluble sugars of plant material for the analysis of carbon isotope composition: a comparison of methods. *Rapid Commun. Mass Spectrom.* 23, 2476–2488 (2009).
88. Monti, A. E., Brugnoli, B., Scartazza, A. & Amaducci, M. T. The effect of transient and continuous drought on yield, photosynthesis and carbon isotope discrimination in sugar beet (*Beta vulgaris* L.). *J. Exp. Bot.* 57, 1253–1262 (2006).
89. Yanochko, G. M. & Yool, A. J. Block by extracellular divalent cations of *Drosophila* Big Brain channels expressed in *Xenopus* oocytes. *Biophys. J.* 86, 1470–1478 (2004).
90. Fetter, K., Van Wilder, V., Moshelion, M. & Chaumont, F. Interactions between plasma membrane aquaporins modulate their water channel activity. *Plant Cell* 16, 215–228 (2004).
91. Liu, K. et al. Conversion of aquaporin 6 from an anion channel to a water-selective channel by a single amino acid substitution. *Proc. Natl. Acad. Sci. USA* 102, 2192–2197 (2005).
92. Shi, L. W. SPSS19.0 Statistical analysis from accident to conversance (in Chinese). *Beijing: Tsinghua Univ. Press.* pp, 88–143 (2012).

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**Author Contributions**

L.L. performed the genetic engineering, in vivo molecular interaction, gas exchange, and chlorophyll fluorescence experiments. H.W. performed the oocyte assay, gene expression, and transgenic plant characterization experiments. H.C. performed the isotopic discrimination experiment. Z.Q. performed the cell pressure probe experiment. H.J., S.T. and D.S. performed the Y2H and protein production experiments. Y.C., F.S. and Z.X. assisted characterization of transgenic plants. J.G., N.K., Q.Y., W.S., J.F.
and H.D. designed the experiments. Q.Y., W.S., J.F. and H.D. supervised and coordinated the research. L.L., H.W., D.S., Q.Y., W.S., J.F. and H.D. analyzed the data and wrote the manuscript.

Additional Information

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