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Rapid Paper

Overexpression of the Barley Aquaporin HvPIP2;1 Increases Internal CO_2 Conductance and CO_2 Assimilation in the Leaves of Transgenic Rice Plants

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The internal conductance for CO_2 diffusion (g_i) and CO_2 assimilation rate were measured and the related anatomical characteristics were investigated in transgenic rice leaves that overexpressed barley aquaporin HvPIP2;1. This study was performed to test the hypothesis that aquaporin facilitates CO_2 diffusion within leaves. The g_i value was estimated for intact leaves by concurrent measurements of gas exchange and carbon isotope ratio. The leaves of the transgenic rice plants that expressed the highest levels of Aq-anti-HvPIP2;1 showed a 40% increase in g_i as compared to g_i in the leaves of wild-type rice plants. The increase in g_i was accompanied by a 14% increase in CO_2 assimilation rate and a 27% increase in stomatal conductance (g_s). The transgenic plants that had low levels of Aq-anti-HvPIP2;1 showed decreases in g_i and CO_2 assimilation rate. In the plants with high levels of Aq-anti-HvPIP2;1, mesophyll cell size decreased and the cell walls of the epidermis and mesophyll cells thickened, indicating that the leaves had become xeromorphic. Although such anatomical changes could partially offset the increase in g_i by the aquaporin, the increase in aquaporin content overcame such adverse effects.

Keywords: Aquaporin — CO_2 assimilation rate — HvPIP2;1 — Mesophyll anatomy — Transgenic rice — Stomatal CO_2 conductance.

Abbreviations: Aq-anti-HvPIP2;1, aquaporins detected by an antibody raised against HvPIP2;1; g_i, internal CO_2 conductance; g_s, stomatal CO_2 conductance; C_a, ambient CO_2 partial pressure; C_c, chloroplast CO_2 partial pressure; C_i, intercellular CO_2 partial pressure; S_c, surface area of chloroplasts facing the intercellular air space; S_{mes}, surface area of mesophyll cells facing the intercellular air space; Tr6322-H, transgenic plants of line 6322 with high levels of Aq-anti-HvPIP2;1; Tr6322-L, transgenic plants of line 6322 with low levels of Aq-anti-HvPIP2;1; Tr6360-L, transgenic plants of line 6360 with low levels of Aq-anti-HvPIP2;1.

Introduction

The resistance to CO_2 diffusion from the atmosphere to chloroplasts is finite and limits leaf photosynthesis. Internal conductance (g_i), i.e., the conductance for CO_2 diffusion from the intercellular space to the chloroplast stroma, is a key factor in CO_2 diffusion because the limitation of photosynthesis by g_i is often greater than the stomatal limitation. The ratio of g_i to the maximum stomatal conductance (g_s) was reported to be 57–89% in the grapefruit and peach (Lloyd et al. 1992), 88% in the chestnut (Lauteri et al. 1997), 60% in temperate deciduous trees (Hanba et al. 2002), and only 50% in temperate evergreen trees (Hanba et al. 2003). Many previous studies have shown that g_i is strongly correlated with CO_2 assimilation rates in a variety of plant species, including annual crops (von Caemmerer and Evans 1991, Evans et al. 1994) and tree species (Lloyd et al. 1992, Loreto et al. 1992, Epron et al. 1995, Hanba et al. 1999, Hanba et al. 2002).

The value of g_i is determined by the conductance in the gas phase (g_{gas}) and that in the liquid phase (g_{liq}). In amphistomatous leaves with high mesophyll porosity, such as tobacco leaves, g_i is determined mostly by g_{liq}, which can be expressed as $g_{liq} = C_{liq} \times S_c$ (Evans et al. 1994), where C_{liq} is conductance per unit of exposed chloroplast surface area, and S_c is surface area of chloroplasts facing the intercellular air space. Therefore, S_c should be one of a determinant of g_i (Evans 1998).

The cell wall and plasma membrane can affect C_{liq} as they impose considerable resistance to CO_2 diffusion (Nobel 1999). Kogami et al. (2001) showed that a thick cell wall decreases g_i in the highland ecotype of *Polygonum cuspidatum*. Evergreen tree species have thick cell walls, which may be a reason for their low g_i (Miyazawa and Terashima 2001). S_c and the thickness of the cell wall, which affect g_i in opposite directions, change concurrently in some cases. In the maple species *Acer rufinerve*, both S_c and cell wall thickness are increased in sun leaves as compared to shade leaves, which offsets the change in g_i (Hanba et al. 2002). These studies have shown that the cell wall imposes considerable resistance to CO_2 diffusion inside leaves.

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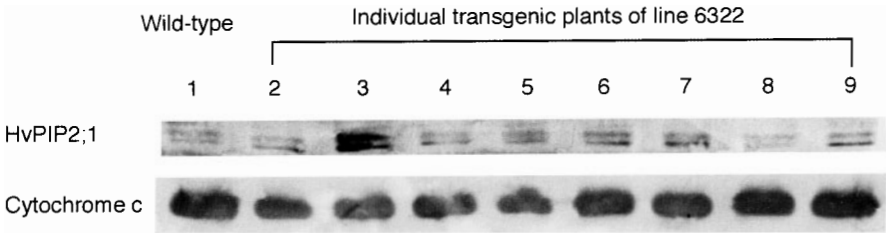

Fig. 1 Western analysis were performed for the leaves of wild-type (lane 1) and the transgenic rice plants (line 6322, lanes 2–9) of the T_2 generation. For one plant, one leaf was used. Protein (20 μg each) from the leaf was subjected to electrophoresis and detected with an anti-HvPIP2;1 antibody or anti-cytochrome c antibody as a loading control. Two bands recognized by anti-HvPIP2;1 antibody were combined in the quantitative analysis (see Materials and Methods).

However, the physiological and biochemical factors that limit CO_2 diffusion across the plasma membrane are not clear. One candidate for such a factor is aquaporin. Aquaporin is a membrane protein that was originally characterized as a water channel through which H_2O can permeate the biological membrane (Tyerman et al. 1999). Some studies in mammalian cells indicated that CO_2 can permeate through the aquaporin AQP1 (Nakhoul et al. 1998, Cooper and Boron 1998), while Yang et al. (2000) raised questions over this interpretation. Recently, Uehlein et al. (2003) reported that the membrane permeability of CO_2 was increased in *Xenopus* oocytes expressing the tobacco aquaporin NtAQP1. However, no measurements of g_i were performed in their studies, so it is not clear whether the increase in membrane permeability actually enhances CO_2 diffusion inside the leaves. The possible role of aquaporin in CO_2 diffusion in higher plants was first examined by Terashima and Ono (2002). They detected a significant decrease of g_i in the presence of $HgCl_2$, an inhibitor of most aquaporins. However, mercurial compounds have non-specific effects on metabolism (Tyerman et al. 1999) and exert a broad range of secondary effects related to regulation of aquaporin activity (Baiges et al. 2002). Therefore, experiments using other compounds must be performed to confirm the role of aquaporin in CO_2 diffusion in higher plants.

In the present study, we produced transgenic rice plants overexpressing a barley aquaporin (HvPIP2;1) to test the hypothesis that the level of the aquaporin is one of the factors determining internal CO_2 diffusion. The transformation may also induce some anatomical changes in the leaves, as the physiological status of the transgenic plants may differ considerably from that of wild-type rice. Because mesophyll anatomy and stomatal distribution exert significant effects on g_i (Parkhurst and Mott 1990, Evans et al. 1994, Syvertsen et al. 1995, Kogami et al. 2001), we also compared the anatomical properties of the transgenic and wild-type rice plants.

Results

Variation in the level of aquaporin in the rice leaves

The transgenic plants used in the present study were T_2 generations of lines 6322 and 6360, which were descendents of the same line of T_0 generation with a high level of Aq-anti-HvPIP2;1. The level of Aq-anti-HvPIP2;1 in the leaves differed among the individual plants of the T_2 generation of line 6322 (Fig. 1). Aq-anti-HvPIP2;1 was also detected in the leaves of the wild-type rice plants (see Materials and Methods). Some of the individual rice plants showed significant increases in leaf Aq-anti-HvPIP2;1 as compared to wild-type plants, while others showed no such increase. The individual transgenic plants of line 6360 showed no increases and even decreases in leaf Aq-anti-HvPIP2;1 levels when compared with wild-type controls (data not shown).

Leaf gas exchange of the wild-type and transgenic rice plants

The rice plants investigated in the present study were classified into four groups according to the level of Aq-anti-HvPIP2;1 in the leaves (Fig. 2A): wild type; transgenic plants of line 6322 with low levels of Aq-anti-HvPIP2;1 (Tr6322-L); transgenic plants of line 6322 with high levels of Aq-anti-HvPIP2;1 (Tr6322-H); and transgenic plants of line 6360 with low levels of Aq-anti-HvPIP2;1 (Tr6360-L). The number of plants in each group varied from 4 to 7. The average level of Aq-anti-HvPIP2;1 in the leaves was 135% higher in Tr6322-H, 33% lower in Tr6322-L, and 12% lower in Tr6360-L than in the wild-type plants, although the decrease of Aq-anti-HvPIP2;1 in Tr6322-L or Tr6360-L was not statistically significant (Fig. 2A). The level of Aq-anti-HvPIP2;1 had a significant impact on the internal CO_2 conductance (g_i) of the leaves (Fig. 2B). Tr6322-H showed 40% higher g_i, while Tr6322-l and Tr6360-L showed 15% and 26% lower g_i than wild-type plants, respectively. Stomatal conductance was significantly increased in Tr6322-H by 26%, while no such increases were observed in Tr6322-L and Tr6360-L (Fig. 2C). The effect of aquaporin on the leaf CO_2 assimilation rate was similar to its effect on g_i (Fig. 2D); the leaf CO_2 assimilation rate increased in Tr6322-H by 14%, but decreased in Tr6322-L and Tr6360-L by 9% and 16%, respectively, as compared to the wild-type plants.

Although stomatal conductance was significantly higher in Tr6322-H than in wild-type plants, the intercellular CO_2 partial pressure (C_i) in Tr6322-H was only slightly higher than that in wild type (Table 1). On the other hand, the chloroplast CO_2 partial pressure (C_c) was much higher in Tr6322-H than in

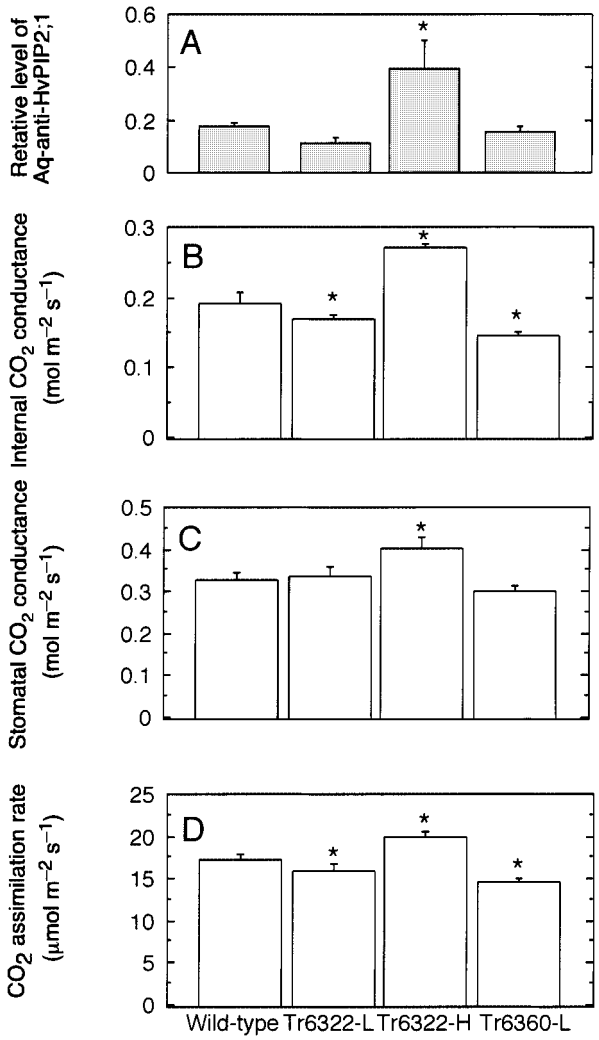

Fig. 2 Relative level of leaf aquaporin detected with an anti-HvPIP2; 1 antibody (Aq-anti-HvPIP2;1) and gas exchange parameters in the rice leaves. Transgenic plants were of the T_2 generations of lines 6322 and 6360. The rice plants were grouped according to the relative level of Aq-anti-HvPIP2;1 using Western analysis (A). The numbers of plants were as follows: wild type, n = 7; Tr6322-H, n = 4; Tr6322-L, n = 6; Tr6360-L, n = 6. The relative level of Aq-anti-HvPIP2;1 was determined for each plant, expressed as density relative to that of the loading control, cytochrome *c*. Gas exchange parameters, such as internal CO_2 conductance (B), stomatal CO_2 conductance (C), and CO_2 assimilation rate (D), were measured in the intact leaves under PPFD of 740 μmol m^{-2} s^{-1}, ambient CO_2 partial pressure of 35 Pa, leaf temperature of 25°C, and vapor pressure deficit of 830±100 Pa. Gas exchange measurements were perfumed for 4–7 different plants. Values are means ± SE. Asterisks (*) above the bars indicate that the means of the transgenic plants are significantly different from those of the wild-type plants (P <0.05).

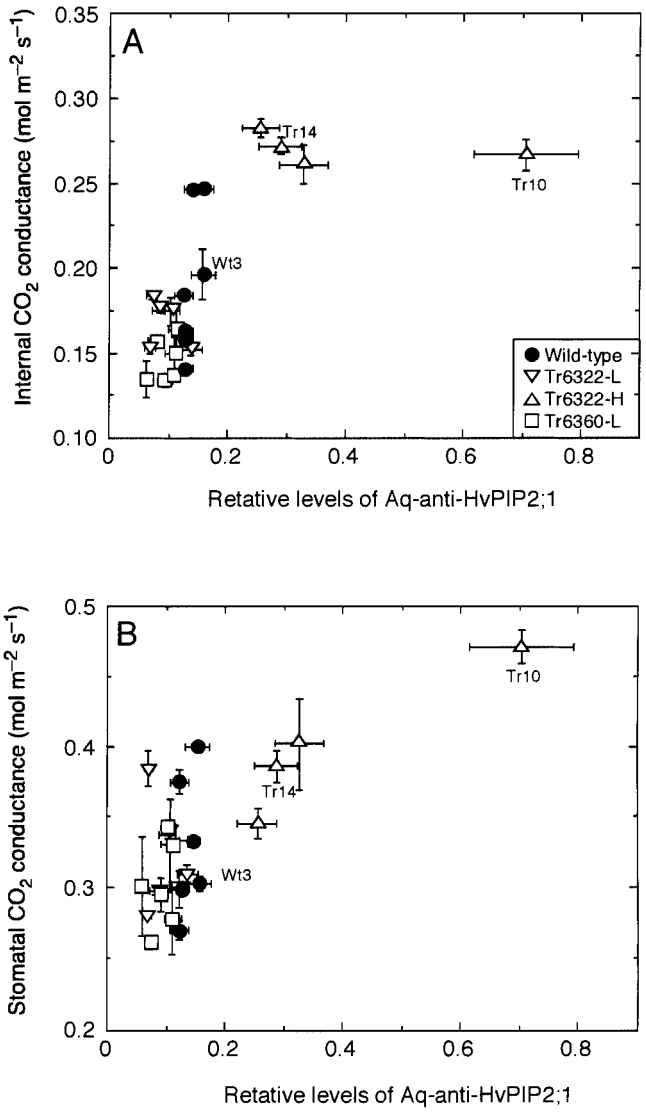

Fig. 3 Relationships between the internal CO_2 conductance (A) or stomatal CO_2 conductance (B) and relative levels of Aq-anti-HvPIP2;1 in individual rice plants. Measurements conditions for gas exchange were described in Fig. 2. Data are shown as means ± SE (n = 2–10) for gas exchange measurements. The relative level of leaf Aq-anti-HvPIP2;1 is expressed as the density relative to that of the loading control cytochrome *c*. For one leaf, three measurements were performed and results are shown as means ± SE.

wild-type plants, as a result of high g_i. Internal conductance was a more important limitation for CO_2 diffusion from the ambient air to the chloroplasts than was stomatal conductance in both transgenic and wild-type plants, as C_c/C_i was much lower than C_i/C_a. Transgenic plants showed lower intrinsic water-use efficiency than did the wild-type plants (Table 1).

Leaf gas exchange and mesophyll anatomy in the individual rice plants

The relationship between the levels of Aq-anti-HvPIP2;1 and g_i was examined in individual plants of the four groups (Fig. 3). The leaves of a transgenic plant designated Tr10 had the highest levels of Aq-anti-HvPIP2;1. However, g_i in these leaves was comparable to those of the other Tr6322-H plants

Table 1 Gas exchange parameters in the leaves of the transgenic and wild-type plants

	C_i (μmol mol^{-1})	C_c (μmol mol^{-1})	C_i/C_a	C_c/C_i	Intrinsic water use efficiency (μmol mol^{-1})
Wild type	290±7	199±17	0.832±0.02	0.687±0.05	54.2±6.4
Tr6322-L	299±4*	205±11	0.853±0.01*	0.686±0.03	47.5±4.3*
Tr6322-H	297±9*	225± 9*	0.843±0.02	0.757±0.02*	50.9±7.6
Tr6360-L	297±8*	196±15	0.849±0.02*	0.661±0.04	48.9±7.2*

Intercellular CO_2 partial pressure (C_i), chloroplast CO_2 partial pressure (C_c), ratio of intercellular to ambient CO_2 partial pressure (C_i/C_a), ratio of chloroplast to intercellular CO_2 partial pressure (C_c/C_i) and ratio of CO_2 assimilation to stomatal conductance (intrinsic water use efficiency) were calculated. Data for each group were obtained from 4–6 plants. All measurements were performed for the different leaves: the numbers of leaves measured were 18 for the wild type, 14 for Tr6322-L, 30 for Tr6322-H and 12 for Tr6360-L. Values are means ± SD (n = 12–30). Asterisks (*) indicate that the means of the transgenic plants are significantly different from those of the wild-type plants (P <0.05). Gas exchange measurements were performed under PPFD of 740 μmol m^{-2} s^{-1} and vapor pressure deficit of 830±100 Pa, and ambient CO_2 partial pressure was kept at 351±3 μmol mol^{-1}. See text for other measurement conditions.

Table 2 Mesophyll anatomy, stomatal density and sizes for three individual plants, shown in Fig. 3

Parameters	Wt3	Tr14, 1.7-fold increase in aquaporin	Tr10, 4.2-fold increase in aquaporin
Mesophyll thickness (μm)	43 ± 2	40 ± 4	42 ± 4
Mesophyll porosity (m^3 m^{-3})	0.26± 0.10	0.14± 0.03*	0.17± 0.03*
S_{mes} (m^2 m^{-2})	19 ± 1	15 ± 2*	15 ± 3*
S_c (m^2 m^{-2})	14 ± 1	14 ± 2	10 ± 2*
Cell wall thickness (μm)	0.13± 0.02	0.13± 0.02	0.25± 0.04*
Stomatal density (no. mm^{-2})	511 ±31	444 ±18*	438 ±37*
Stomatal size (μm)	19.40± 0.40	12 ±0.04*	13.10± 0.70*

Data were obtained from five leaves for each plant. The relative level of aquaporin, detected with an antibody to HvPIP2;1, was increased by 1.7-fold in Tr14 and 4.2-fold in Tr10 as compared to wild type (Wt3). The length of guard cells are shown as means ± SD (n = 20). Otherwise, data are means ± SD (n = 5). Asterisks (*) indicate that the means of the transgenic plants are significantly different from those of the wild-type plants (P <0.05).

(Fig. 3A). On the other hand, Tr10 had a higher stomatal CO_2 conductance than the other Tr6322-H plants (Fig. 3B). Tr10 had thicker mesophyll cell walls than a Tr6322-H plant with 1.7-fold its aquaporin level, designated Tr14 (Table 2). The surface area of chloroplasts (S_c) was lower in Tr10 than in Tr14. Cell wall thickness and S_c differed between Tr10 and Tr14, but there were no significant differences between these two lines in mesophyll anatomy, stomatal density, or stomatal size. The two transgenic plants, Tr10 and Tr14, had lower mesophyll porosity, lower stomatal density, and smaller stomatal size than did the wild-type plant Wt3.

Leaf anatomy of the wild-type and transgenic rice plants

Several anatomical parameters of the leaves were quantified and compared between the wild-type plants and transgenic plants at the expanding and mature stages (Fig 4). Mesophyll porosity decreased significantly with leaf development in Tr6322-H (Fig. 4A). In Tr6322-H, significant development of collenchyma cells outside the vascular bundle was observed at the mature stage, while such development of collenchyma was less marked in wild-type and Tr6360-L (Fig. 4B). The surface area of chloroplasts facing the intercellular air spaces (S_c) was similar between the wild-type and transgenic plants at the ma-

Table 3 Stomatal density, stomatal size, and Rubisco level in the leaves of wild-type and the transgenic line 6322 plants

	Stomatal density (no. mm^{-2})	Stomatal size (μm)	Level of Rubisco (g m^{-2})
Wild type	465±49	15.5±3.5	2.02±0.7
Tr6322-L	333±72*	13.4±0.1	1.77±0.6
Tr6322-H	439±55	12.9±1.2	2.41±0.8

Data were obtained from three wild type, one Tr6322-L, and four Tr6322-H plants. Measurements were repeated for one leaf from each individual plant. Lengths of the guard cells were measured in 20 stomata selected at random, and stomatal distribution was estimated for five replicates for each leaf. Rubisco level was estimated by SDS-PAGE and CBB-staining based on four repeated measurements for each leaf. Values are means ± SD. Asterisks (*) indicate that the means of the transgenic plants are significantly different from those of the wild-type plants (P <0.05).

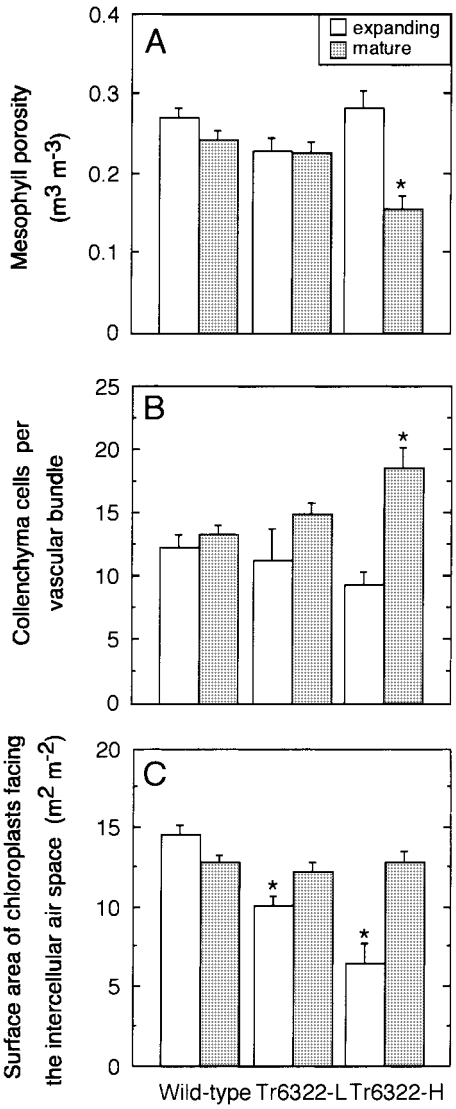

Fig. 4 Mesophyll anatomy were quantified for the wild-type and transgenic plants of line 6322, Tr6322-H, and Tr6322-L at the expanding (10 d old, hollow bars) and mature (46–50 d old, solid bars) stages. (A) Leaf mesophyll porosity. (B) Number of collenchyma cells per vascular bundle. (C) Surface area of chloroplasts facing the intercellular air spaces. Data were obtained from one plant for expanding leaves (n = 5) and from 4–7 plants for the mature leaves (n = 19–26). Values are means ± SE. Asterisks (*) above the bars indicate that the means of the transgenic plants are significantly different from those of the wild-type plants (P <0.05). Statistical analysis was performed separately for expanding and mature leaves.

ture stage (Fig. 4C). Stomatal density and stomatal size were reduced in Tr6360-L as compared to the wild-type controls (Table 3). Although the differences were not statistically significant, Rubisco content was slightly higher in Tr6322-H, but it was slightly lower in Tr6322-L than in the wild type.

Leaf mesophyll anatomy of Tr10 was compared to that of Wt3 (Fig. 5). Tr10 had a thick cuticle and high mesophyll cell density (Fig. 5A, B), well-developed collenchyma, and thicker bundle sheath outer cell walls (Fig. 5C, D) than did Wt3. The mesophyll cell walls of Tr10 were much thicker than those of Wt3 (Fig. 5E, F).

Discussion

Increased aquaporin levels enhanced internal CO_2 conductance

The level of aquaporin detected in the leaves by the HvPIP2;1 antibody (Aq-anti-HvPIP2;1) was strongly related to g_i (Fig. 2A, B). These results indicate that HvPIP2;1 has a role in CO_2 diffusion inside rice leaves. The most likely effect is facilitation of CO_2 diffusion or permeability across the plasma membrane of mesophyll cells, given that HvPIP2;1 is an aquaporin in the plasma membrane-type subfamily (Katsuhara et al. 2002). Recently, Uehlein et al. (2003) suggested that tobacco aquaporin NtAQP1 facilitates membrane CO_2 transport in tobacco leaves, although they did not measure g_i in the leaves. The present results more directly demonstrate the increase in g_i with increases in aquaporin level.

Some of the transgenic rice plants showed lower levels of leaf Aq-anti-HvPIP2;1 than did the wild-type controls (Fig. 1). This unexpected suppression of aquaporin in the leaves might have been due to gene silencing, which is observed frequently in transgenic plants (Meyer and Saedler 1996, Fagard and Vaucheret 2000). Either transcriptional gene silencing, including transgene methylation (Finnegan et al. 1998, Vaucheret and Fagard 2001), or post-transcriptional gene silencing resembling to RNA interference (Vaucheret et al. 2001) might have occurred in these plants.

The increase in aquaporin enhanced CO_2 assimilation rate

The level of leaf Aq-anti-HvPIP2;1 had a significant effect on the leaf CO_2 assimilation rate (Fig. 2D). This observation supports the reports of Aharon et al. (2003) and Uehlein et al. (2003) that overexpression of the aquaporin PIP1b or NtAQP1 enhanced the rate of leaf CO_2 assimilation in tobacco leaves. The increase in the CO_2 assimilation rate in Tr6322-H may be attributed to an increase in the CO_2 partial pressure at the chloroplast site, C_c (Table 1). The increased C_c was mostly due to increased internal conductance (g_i) rather than increased stomatal conductance (g_s), because Tr6322-H had a C_c/C_i value 10% higher than that of wild-type plants, while the C_i/C_a values were similar between the two (Table 1). This suggests that the enhancement of the CO_2 assimilation rate, which was observed in the aquaporin-overexpressing plants in the present study and in previous studies (Aharon et al. 2003, Uehlein et al. 2003), may be partly affected by the increase in g_i. On the other hand, the low CO_2 assimilation rate in Tr6322-L was not directly related to the C_c level; both g_i and CO_2 assimilation rate decreased, resulting similar C_c level in Tr6322-L compared to that in wild type.

Another factor, changes in Rubisco content, also attributed to the changes in CO_2 assimilation rate in the transgenic

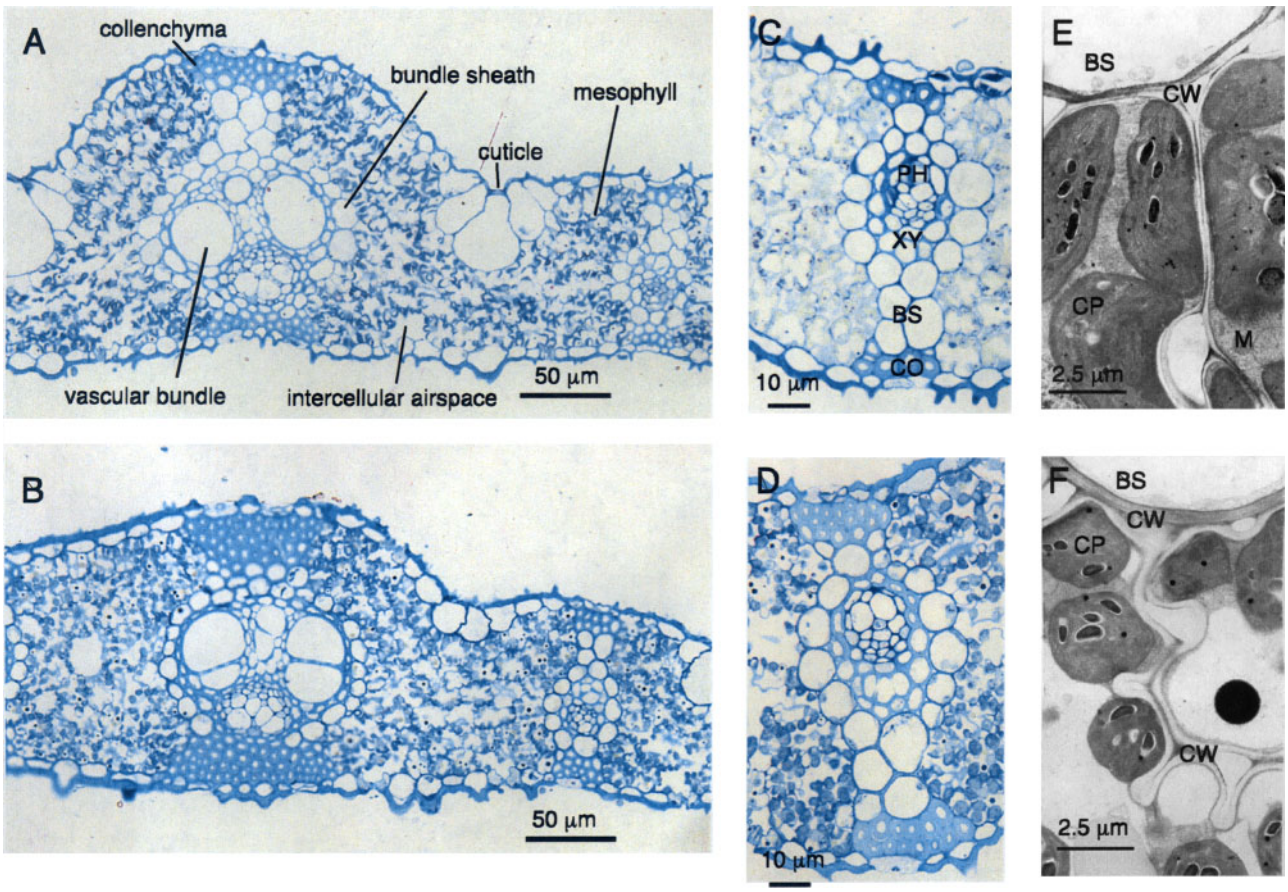

Fig. 5 Leaf mesophyll and vascular bundle anatomy of the rice leaves. Light micrographs of leaf sections were taken at ×400 magnification for wild-type rice plants, Wt3 (A), and high aquaporin transgenic plants, Tr10 (B). The levels of leaf Aq-anti-HvPIP2;1, internal CO_2 conductance, and stomatal CO_2 conductance of these plants are plotted in Fig. 3. Light micrographs of vascular bundles were taken at ×1,000 magnification for Wt3 (C) and Tr10 (D). Electron micrographs of vascular bundle cells and adjacent mesophyll cells were taken at ×4,000 magnification for Wt3 (E) and Tr10 (F). PH, phloem; XY, xylem; BS, bundle sheath; CO, collenchyma, CW, cell wall; CP, chloroplasts.

plants. The increase or decrease of CO_2 assimilation rate in Tr6322-H or Tr6322-L (Fig. 2D) was related to the slight increase or decrease in Rubisco content in Tr6322-H or Tr6322-L (Table 3), although the changes in Rubisco content were not statistically significant. The changes in CO_2 assimilation rate in Tr6322-H and Tr6322-L may be combined results from the changes in both g_i and Rubisco content.

Effect of aquaporin on stomatal conductance and water use efficiency

High internal CO_2 conductance (g_i) was accompanied by high stomatal CO_2 conductance (g_s) in the transgenic rice plants with high levels of leaf Aq-anti-HvPIP2;1, Tr6322-H (Fig. 2C). This result supports those of many previous studies showing a strong correlation between g_i and g_s (Loreto et al. 1992, Lauteri et al. 1997, Loreto et al. 2003, Hanba et al. 2003). The mechanism of the strong relationship between g_i and g_s is not well understood. Uehlein et al. (2003) speculated that the increase in CO_2 permeability by aquaporin is related to the signal regulating stomatal guard cells, resulting in high g_s in transgenic tobacco plants overexpressing NtAQP1.

The increase in g_s in Tr6322-H (Fig. 2C) caused high water loss from the leaves. Furthermore, intrinsic water use efficiency was slightly decreased in Tr6322-H (Table 1), indicating that the increase in leaf Aq-anti-HvPIP2;1 enhanced water loss from the leaves to a greater extent than the CO_2 assimilation rate. This result suggests that Tr6322-H is more susceptible to water stress than are wild-type plants. Aharon et al. (2003) observed a significant increase in transpiration and faster wilting of transgenic tobacco plants that overexpressing the aquaporin PIP1b and concluded that the overexpression may not have any beneficial effect under drought stress, which is in agreement with our findings.

Increased water loss in the high-aquaporin plants (Tr6322-H) induced changes in mesophyll anatomy

Leaf development may have been affected by water deficit in Tr6322-H due to increased water loss, although these plants were grown under well-watered conditions. Mature

leaves of Tr6322-H had a thicker cuticle, higher mesophyll cell density, and more developed collenchyma (Fig. 4A, B) than did the leaves of the wild-type plants. All these features are known to be associated with water stress (Esau 1965). However, these features were not observed in the expanding leaves of Tr6322-H (Fig. 4A, B). This suggests that the anatomical changes in Tr6322-H were not due to inheritance but to acclimatization responses to the physiological or water status of the plants.

These changes in leaf anatomy probably affected g_i in turn. The low mesophyll porosity potentially reduces g_i in the thick and hypostomatous leaves (Parkhurst 1994), although CO_2 diffusion in gas phase affects little on g_i in hypostomatous leaves such as *Rosa rubiginosa* (Genty et al. 1998). In the present study, the decreasing mesophyll porosity might not affect g_i, because rice leaves are thin and amphistomatous. In the mature leaves, the surface area of chloroplasts facing the intercellular air space (S_c), one of the determining factors for g_i (Evans et al. 1994), did not differ between the transgenic and wild-type plants (Fig. 4C). Thus, we concluded that changes in these anatomical characteristics were not responsible for the differences in g_i between Tr6322-H, Tr6322-L, and wild-type plants.

Internal CO_2 diffusion and mesophyll anatomy in plants with an extremely high level of aquaporin

The relationship between the level of Aq-anti-HvPIP2;1 and g_i for individual rice plants (Fig. 3A) suggests that the enhancement of g_i by Aq-anti-HvPIP2;1 might be offset when the level of Aq-anti-HvPIP2;1 exceeds a certain threshold. The transgenic plants with the highest level of Aq-anti-HvPIP2;1 (Tr10) had the highest stomatal CO_2 conductance among all of the plants investigated (Fig. 3B), resulting in highest water deficit. Tr10 had thicker mesophyll cell walls than did wild-type plants or transgenic plants with a 1.4-fold increase in the level of aquaporin (Tr14, Table 2). As we suggested previously (Kogami et al. 2001, Miyazawa and Terashima 2001, Hanba et al. 2002), thick cell walls decrease g_i. Therefore, the 90% increase in the thickness of mesophyll cell walls in Tr10 (Table 2) might decrease the Aq-anti-HvPIP2;1-induced increase in g_i.

Conclusions

We first demonstrated that the overexpression of HvPIP2;1, which increased the level of Aq-anti-HvPIP2;1 by 135%, enhanced the internal CO_2 conductance in intact rice leaves by 40%. This result confirmed the role of aquaporin in CO_2 diffusion inside plant leaves. The abundance and/or activity of aquaporins would decrease in water- or salt-stressed plants (Tyerman et al. 2002), so the significant reduction in g_i in salt-stressed olive leaves (Loreto et al. 2003) may be due to the reduction in CO_2 permeability through aquaporins. The increase in Aq-anti-HvPIP2;1 was related to the 14% increase in CO_2 assimilation rate in the present study (Fig. 2D); thus these plants might have higher growth rates than those of wild-type controls if they have a sufficient water supply. However, it should be noted that these plants are susceptible to water stress, as overexpression of aquaporin induces an increase in transpiration. The results of the present study suggest that an anatomical alteration is induced by water stress, which offsets internal CO_2 conductance, if the level of aquaporin exceeds a certain threshold. Further studies such as antisense experiments are needed to understand the quantitative effects of increases in aquaporin on anatomical changes and internal CO_2 conductance.

Materials and Methods

Plant transformation and growth conditions

Transgenic rice plants (*Oryza sativa* var. Kinuhikari) overexpressing the barley aquaporin HvPIP2;1 were produced as described previously (Katsuhara et al. 2003). Briefly, the first intron of the castor bean catalase gene was introduced to enhance foreign gene expression in rice (Tanaka et al. 1990). The whole coding region of HvPIP2;1, which is a barley aquaporin belonging to the plasma membrane subfamily (Katsuhara et al. 2002), was inserted downstream of the 35S-promoter to construct the expression vector P35S·Int·HvPIP2;1·Nos. Northern analysis of T_0 cells (callus) showed high levels of HvPIP2;1 expression (data not shown). The selection of transgenic callus, regeneration of transgenic rice plants, and their growth in culture bottles were performed as described previously (Shimamoto et al. 1989). Regenerated T_0 and T_1 plants were grown to maturity and seeds were obtained by self-pollination. T_2 plants were used in all experiments.

The rice plants of the T_2 generation were transplanted into Wagner pots (1/500 area) filled with soil (1 : 1 mixture of paddy soil and humus), fertilized with 400 mg/pot each of N, P_2O_5, and K_2O, and grown in a controlled-environment growth chamber (MLR-350H, Sanyo, Tokyo, Japan) for 80 d at 25°C, 60% relative humidity, 150 μmol m^{-2} s^{-1} PPFD, and a 14-h photoperiod. All plants grew well under these conditions.

HvPIP2;1 expression measurements

The plant leaves were frozen in liquid N_2 and homogenized in 3-fold (v/w) grinding buffer containing 0.3 M sucrose, 8 mM EDTA, 4 mM DTT, 2 mM phenylmethylsulfonylfluoride, and 50 mM Tris. The homogenate was centrifuged at 10,000×*g* for 10 min at 4°C, and the supernatant containing soluble proteins and microsomal membrane proteins was recovered. The protein concentration was determined by the Bradford method. To solubilize membrane proteins, the supernatant was mixed with the same volume of a solubilization buffer [60% (w/v) sucrose : 10% (w/v) lithium lauryl sulfate : H_2O, 2 : 2 : 1, including 18.4 mg ml^{-1} DTT].

The level of HvPIP2;1 was determined by Western analysis as described previously (Katsuhara et al. 2002), using cytochrome c as an internal control. Briefly, polyclonal antibodies were raised against a synthetic oligopeptide, AAPQGGEFSSKDYS, corresponding to the N-terminal region of HvPIP2;1. In barley, the antibody recognized two bands, which might represent aquaporin isoforms with similar epitopes (Katsuhara et al. 2002). The antibody also recognized two bands in the transgenic rice plants overexpressing HvPIP2;1.

Cross-reactivity on Western analysis was observed in the wild-type rice leaves, although no sequence identical to the N-terminal region of HvPIP2;1 (AAPQGGEFSSKDYS) was found in the deduced amino acid sequences from the rice DNA sequence data. A similar rice sequence was found in part of a BAC clone (AP006168), SAPEGGEFSAKDYT, showing 71% identity to the barley query sequence. The closest match in the rice EST data base was AAAEGGEYMAKDYS

(64% identity). These similar sequences might be expressed in the control rice leaves and cross-react with the antibody.

Gas exchange measurements and estimation of internal CO_2 conductance

Gas exchange measurements were performed at PPFD of 740 μmol m^{-2} s^{-1}, ambient CO_2 partial pressure of 35 Pa, and leaf temperature of 25°C, using a laboratory-constructed system described by Hanba et al. (1999). Gas exchange parameters were calculated according to the method of von Caemmerer and Farquhar (1981). Internal CO_2 conductance (g_i), the CO_2 transfer conductance from the substomatal cavity to the carboxylation site in the stroma, was estimated for intact leaves by concurrent measurement of gas exchange and carbon isotope ratio (Hanba et al. 2002). The isotope method is considered the most reliable for the estimation of g_i. In the present study, g_i was calculated using the equation reported by Scartazza et al. (1998):

$$g_i = \frac{(b - a_i)A/C_a}{(\Delta_i - \Delta) - f\Gamma^*/C_a} \quad \text{eqn (1)},$$

where Δ is the actual carbon isotope discrimination, Δ_i is the expected carbon isotope discrimination assuming infinite g_i, A is assimilation rate, C_a is CO_2 partial pressure in the ambient air, a_i is carbon isotope discrimination during CO_2 diffusion/hydration into water (1.8‰), and b is carbon isotope discrimination caused by carboxylation by Rubisco and PEP carboxylase (30‰). The symbols f and Γ^* represent discrimination with photorespiration and a CO_2 compensation point without dark respiration, respectively. Here, we assumed that f was negligible (von Caemmerer and Evans 1991). Measurements were repeated twice for each leaf sample at PPFD of 740 μmol m^{-2} s^{-1}. Δ was calculated from carbon isotope ratios of CO_2 in air leaving and entering the gas exchange chamber. CO_2 samples were collected using dry ice-ethanol and liquid nitrogen traps. The carbon isotope ratio was determined with an isotope mass spectrometer (Finnigan MAT 252, Bremen, Germany). Analysis of the carbon isotope ratio was performed as described by Hanba et al. (1999).

The CO_2 partial pressure in chloroplasts (C_c) was calculated using the equation:

$$C_c = C_i - A/g_i \quad \text{eqn (2)},$$

where C_i is the partial pressure of CO_2 in the intercellular air spaces.

Gas exchange measurements were performed in January 2002 for expanding leaves (10 d old) of line 6322 and in May 2002 for the mature leaves (46–50 d old) of line 6322 and 6360; measurements were repeated in October 2002 for mature leaves (46–50 d old) of line 6322.

Analysis of leaf mesophyll anatomy

Mesophyll anatomy, stomatal density, and size were analyzed in the leaf parts 5 cm from the tip of the leaf blades. For leaf mesophyll anatomy, leaf samples measuring 1×3 mm were fixed in 2.5% glutaraldehyde and 2% osmium tetroxide, and embedded in Spurr's resin (Spurr 1969). Transverse sections were stained with 1% toluidine blue solution, and light micrographs were taken with a digital camera (DP11, Olympus, Tokyo, Japan). Anatomical characteristics were determined from the digitized images of the micrographs at ×1,000 magnification. Surface areas of mesophyll cells (S_{mes}) and chloroplasts (S_c) exposed to intercellular air spaces per unit leaf area were estimated for 700-nm-thick transverse sections as described previously (Hanba et al. 2002). For the measurement of cell wall thickness, ultrathin 70-nm-thick sections were stained with lead citrate and uranyl acetate, and examined at a final magnification of ×4,000 using an electron microscope (Hitachi 7100, Tokyo, Japan). For stomatal analysis, a replica of the abaxial epidermis was obtained from each leaf disk (1 cm^2) using nail varnish. The density of stomata was counted for five replicas. The length of guard cells was measured for 20 stomata selected at random.

Measurements of Rubisco

Two leaf disks (0.5 cm^2 × 2) were obtained from the central part of the lamina and were homogenized immediately at 4°C in 50 mM HEPES buffer (pH 7.5) containing 0.2% Triton X-100, 0.7% 2-mercaptoethanol, 2 mM monoiodoacetic acid, 25% glycerol, 6% lithium lauryl sulfate, 1% polyvinyl polypyrrolidone, and 1 mM phenylmethylsulfonylfluoride. The homogenate was immediately centrifuged, and the supernatant (0.2 cm^3) was boiled for 5 min in Laemmli buffer (Laemmli 1970). Proteins in the supernatants were separated by SDS-PAGE (14%), and the gels were stained with a Coomassie brilliant blue G-250 (Bio-safe Coomassie, Bio-Rad, CA, U.S.A.). The level of Rubisco subunits was determined spectrophotometrically by scanning the gel with a scanner (GT7600U, Epson, Tokyo, Japan). The data were processed using the software Densitograph (ATTO, Tokyo, Japan). The level of Rubisco was determined using BSA as a standard.

Statistical analysis

The difference of mean values between transgenic and wild-type plants was analyzed using analysis of variance (ANOVA) and tested by Fisher's PLSD test.

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