Increased leaf photosynthesis caused by elevated stomatal conductance in a rice mutant deficient in SLAC1, a guard cell anion channel protein

Kensuke Kusumi¹*, Shoko Hirotsuka¹, Toshiharu Kumamaru² and Koh Iba²

¹ Department of Biology; Faculty of Science, Kyushu University, Fukuoka 812–8581, Japan
² Faculty of Agriculture, Kyushu University, Fukuoka 812–8151, Japan

*To whom correspondence should be addressed. E-mail: kkususcb@kyushu-u.org

Received 2012; Revised 19 June 2012; Accepted 6 July 2012

Abstract

In rice (Oryza sativa L.), leaf photosynthesis is known to be highly correlated with stomatal conductance; however, it remains unclear whether stomatal conductance dominantly limits the photosynthetic rate. SLAC1 is a stomatal anion channel protein controlling stomatal closure in response to environmental [CO₂]. In order to examine stomatal limitations to photosynthesis, a SLAC1-deficient mutant of rice was isolated and characterized. A TILLING screen of N-methyl-N-nitrosourea-derived mutant lines was conducted for the rice SLAC1 orthologue gene Os04g0674700, and four mutant lines containing mutations within the open reading frame were obtained. A second screen using an infrared thermography camera revealed that one of the mutants, named slac1, had a constitutive low leaf temperature phenotype. Measurement of leaf gas exchange showed that slac1 plants grown in the greenhouse had significantly higher stomatal conductance (gₛ), rates of photosynthesis (A), and ratios of internal [CO₂] to ambient [CO₂] (Cᵢ/Cₐ) compared with wild-type plants, whereas there was no significant difference in the response of photosynthesis to internal [CO₂] (A/Cᵢ curves). These observations demonstrate that in well-watered conditions, stomatal conductance is a major determinant of photosynthetic rate in rice.

Key words: Anion channel, carbon dioxide, mutant, Oryza sativa, photosynthesis, SLAC1, stomatal conductance.

Introduction

Stomatal pores in the epidermis provide gates for two very important plant processes, photosynthesis and transpiration. Terrestrial plants open and close stomata to regulate CO₂ uptake and water evaporation in response to environmental and biochemical stimuli. In principle, increases in stomatal conductance (gₛ), which regulates gas exchange (CO₂ and water), can allow plants under well-watered growth conditions to increase their CO₂ uptake and subsequently enhance photosynthesis. However, the relationship between stomatal conductance, CO₂ uptake, and photosynthesis is not so simple in nature. Since a large number of environmental factors affect stomatal aperture (Willmer and Fricker, 1996), the contribution of stomatal regulation to photosynthesis can vary depending on the plant species.

In rice (Oryza sativa L.), stomatal aperture as well as conductance is strongly correlated with leaf photosynthesis (Ishihara and Saito, 1987; Hirasawa et al., 1988). Whereas photosynthesis by rice leaves is also influenced by other factors, such as leaf nitrogen content (Ishihara et al., 1979; Makino et al., 1988) and content of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Makino et al., 1987), stomatal conductance is co-dominantly correlated with the rate of leaf photosynthesis. For example, an indica rice variety Takanari is known as a high-yielding variety that has higher grain yield and dry matter production compared with common rice varieties even at the same rate of nitrogen application (Xu et al., 1997). It has been reported that the high-yielding capacity of Takanari is caused by
higher stomatal conductance that was responsible for a high rate of leaf photosynthesis (Xu et al., 1997; Taylaran et al., 2011). Habataki is also a high-yielding rice variety and exhibited higher leaf photosynthetic capacity that paralleled its higher stomatal conductance (Adachi et al., 2011). Previous studies have shown that varietal differences in stomatal conductance are positively correlated with leaf conductance (Maruyama and Tajima, 1990) as well as with the rate of photosynthesis (Ohsumi et al., 2007). While other factors, such as cuticular conductance and leaf boundary layer conductance, also contribute to whole leaf conductance, these studies show a high correlation between leaf conductance and stomatal conductance. However, it is unclear whether stomatal conductance dominantly limits the photosynthesis.

The SLAC1 gene was isolated from Arabidopsis and encodes an anion transporter protein that is localized in the membrane of stomatal guard cells (Negi et al., 2008; Vahisalu et al., 2008). SLAC1 protein is essential for stomatal closure in response to [CO2], and SLAC1-deficient mutants exhibited a constitutive low leaf temperature phenotype caused by increased respiration. SLAC1 expression specifically occurred in stomatal guard cells, and only stomatal closure was directly affected by the SLAC1 deficiency. At least in the growth chamber, the apparent phenotype of SLAC-deficient mutants was restricted to the guard cells, and no differences in tissue organization were detectable compared with wild-type plants. A SLAC1-deficient plant, therefore, would be a useful model for studying the influence of stomatal conductance on gas exchange and photosynthesis under varying environmental conditions.

In this study, a rice SLAC1-deficient mutant, designated slac1, was generated and characterized. In the slac1 mutant, stomata were constitutively opened and CO2 assimilation concomitantly increased. Phenotypic observations and measurement of gas exchange in response to [CO2] changes suggest that in rice SLAC1 is involved in stomatal closure, as was observed in Arabidopsis, and that stomatal conductance limits photosynthesis in well-watered growth conditions.

Materials and methods

Plant material and treatments

Seeds of wild-type rice (O. sativa L. cv. Taichung 65) and the slac1 mutant were sown on a commercial soil mixture (SunAgro Baiyodo) in plastic pots and placed in a growth chamber (Advantec IS-2300) maintained at a temperature of 25 °C with constant fluorescent light (150 μmol m−2 s−1 at 25 °C, 50% relative humidity), and then transferred to an environmental chamber (constant white light of 200 μmol m−2 s−1 at 25 °C, 40% relative humidity), equipped with an automatic CO2 control unit (Koito FR-SP). After 2h of adaptation to a low [CO2] (100 ppm), thermal images were captured under different CO2 conditions.

Measurement of C and N concentrations

The concentrations of organic carbon and nitrogen in leaves were measured using a thermal video system (Nippon Avionics TVS-8500) as previously described (Hashimoto et al., 2006). Plants were grown on a soil mixture (Baiyodo; SunAgro, Japan) for 14 d in a growth chamber (constant white light of 150 μmol m−2 s−1 at 25 °C, 50% relative humidity), and then transferred to an environmental chamber (constant white light of 200 μmol m−2 s−1 at 25 °C, 40% relative humidity), equipped with an automatic CO2 control unit (Koito FR-SP). After 2h of adaptation to a low [CO2] (100 ppm), thermal images were captured under different CO2 conditions.

Gas exchange measurements

The photosynthetic rate (A), leaf conductance (g), and intercellular [CO2] (C) were measured using a portable gas exchange fluorescence system (Walz GFS-3000). For these experiments, wild-type and slac1 mutant plants were grown on soil in 18 litre pots under greenhouse conditions. Measurements were made on the middle portion of the sixth or seventh leaf of seedlings at the 7.5 stage (Itoh et al., 2005) between 11:00 h and 13:00 h on 3 August. The leaf cuvette environment was controlled at a photosynthetically active radiation (PAR) of 1200 μmol m−2 s−1, relative humidity 60%, leaf temperature 25 °C, and [CO2] of 380 ppm, unless otherwise noted. To analyse the photosynthetic activity without the effect of stomatal conductance, the photosynthetic rate at the same C, was also measured. The C value was calculated as described (von Caemmerer and Farquhar, 1981).

Stomatal density measurements

Fully expanded flag leaves of wild-type and slac1 mutant seedlings grown on soil in the greenhouse were sampled. A drop of instant glue (Konishi Aron Alpha) was applied to a glass slide, and the middle portion of a sampled leaf was pressed on the glue for ~30 s. The leaf was removed and the imprint on the glass slide was observed under a light microscope. Stomatal density was calculated using ×100 magnification and a 0.5 mm×0.5 mm mask.

Results

SLAC1 orthologue gene in rice

SLAC1 was originally cloned from Arabidopsis and proposed to encode a guard cell anion channel (Negi et al., 2008; Saji et al., 2008; Vahisalu et al., 2008) that is related to yeast and
bacterial malate transporters. SLAC1 belongs to a small gene family with five members in Arabidopsis, whereas rice has nine homologous proteins (Fig. 1A) (Vahisalu et al., 2008). Among them, Os04g0674700 (MSU accession: LOC_Os04g57850.1) is the closest rice homologue of the Arabidopsis SLAC1 gene At1g12480 (Fig. 1A). Both Os04g0674700 and At1g12480 have three exons, and the positions of the introns within the DNA sequence are fully conserved (Fig. 1B). The putative amino acid sequence of Os04g0674700 shares 64% identity and 91% similarity with that of At1g12480 (Fig. 2). The previously reported phosphorylation sites in Arabidopsis SLAC1, S59, S86, S113, and S120 (Geiger et al., 2010; Du et al., 2011), are all conserved in Os04g0674700 (Fig. 2). Furthermore, it was confirmed that the predicted 3D structure of Os04g0674700 overlapped greatly with that of the already known SLAC1 orthologue H. influenzae TehA (Supplementary Fig. S1 at JXB online), as was reported in Arabidopsis (Chen et al., 2010; Du et al., 2011). When the amino acid sequence of the SLAC1 middle transmembrane region (F201–F515) was used to query the PDB database, the Phyre2 server exhibited 100% confidence with the structure of template TehA (PDB ID: 3M72). The spatial distribution of 10 transmembrane helices were well conserved between SLAC1 and TehA, and helical hairpins are arranged with a quasi 5-fold symmetry as reported in Arabidopsis SLAC1. No other rice candidate genes with high sequence similarity and structure were found. This was, therefore, designated as SLAC1.

Stomatal development in rice epidermis occurs in differentiating young leaves. Successive leaves develop in an ordered manner, and the number of growing leaves in one shoot is practically constant throughout plant development. When a new leaf emerges, there usually are five younger leaves at different developmental stages. These leaves, which are concealed by the older leaf sheaths, go through successive stages of development, from P0 (leaf founder) to P5 (emerging leaf; Fig. 3A) (Nemoto and Yamazaki, 1993; Itoh et al., 2005; Kusumi et al., 2010a). An anatomical study revealed that the stomatal cell row on the leaf epidermis is determined at the P3 stage and stomatal cells appear during the P4 stage (Itoh et al., 2005). Therefore, the expression of SLAC1 was examined by qRT-PCR in developing leaves (Fig. 3B). The expressions of HT1, which encodes a protein kinase involved in CO2-dependent stomatal movement, and STOMAGEN, which encodes a positive regulator of stomatal density, were also measured (Hashimoto et al., 2006; Sugano et al., 2010). qRT-PCR analyses suggested that transcripts of SLAC1 and STOMAGEN had already abundantly accumulated in the shoot base tissue containing developing leaves at the P0–P3 stages, and levels gradually decreased during the P4 stage. Expression of HT1 was also observed in the early P4 stage but peaked during the late P4 stage. Expression of MUTE and FAMA, basic helix–loop–helix (bHLH)-type transcription factors regulating early stomatal development, was reported to occur mostly during the same stages, P1–P4 (Liu et al., 2009), a finding consistent with the present results. The parallel expression of SLAC1 with other genes involved in stomatal formation suggests the involvement of SLAC1 in rice stomatal development.

Screening for a SLAC1-deficient mutant

In order to find a SLAC1-deficient mutant of rice, a screening procedure called TILLING (Targeting Induced Local Lesions IN Genomes), whereby pinpoint mutations in individual genes can be detected using a PCR-based assay, was used (Till et al., 2003). A modified TILLING method with non-fluorescent primers and a rice mutant library generated by treatment of fertilized egg cell with the chemical mutagen N-methyl-N-nitrosourea (MNU) was used (Suzuki et al., 2008). For the first screening, the pooled M2 DNA samples from 874 lines (one line = six M2 plants) of Taichung 65 and Kinmaze were used. To identify the mutant line in the pool, DNA samples mixed from individual tissues of six M2 plants were used for amplifying the SLAC1 gene region. To amplify the coding region of SLAC1, two primer sets were designed from the sequence of Nipponbare obtained from the Rice Annotation Project (RAP) database (build 5.0; http://rapdb.dna.affrc.go.jp/) (Fig. 1B). Seven mutations, G37A, C493T, G576A, C896T, C1161T, G1208C, and C1795T, were detected by the TILLING screen (Table 1). Among them, G1208C was a transversion mutation and the others were transition mutations. In reference to the gene annotation (AK106615), the G576A mutation occurred in the intron, and the other mutations occurred in exons and caused amino acid changes (Table 1, Fig. 2). Mutations C896T, C1161T, and G1208C were detected simultaneously in line 5S54, and the other four mutations were found as single mutations in individual lines, 15S28, 17S62, 2S66, and 18S59 (Table 1).
In *Arabidopsis*, SLAC1 deficiency caused CO₂-insensitive, constitutive low-temperature phenotypes (Negi et al., 2008), because leaf temperature correlates with the amount of transpired water via stomatal openings due to evaporative cooling (Merlot et al., 2002). Therefore, an additional screen was next developed using infrared thermal imaging to identify mutants that displayed phenotypes similar to *Arabidopsis* SLAC1-deficient mutants. M3 plants of the wild type (Taichung 65) and five mutant lines were grown for 2 weeks in a growth chamber, and were then subjected to analysis by thermography. Since a high [CO₂] causes stomatal closing, there was measurable leaf warming in wild-type plants. Among the five mutant lines, 17S62 and 5S54 exhibited lower leaf temperatures than the wild type and the other mutant lines under both high and low [CO₂]. Since the phenotype of 5S54 was stable and even much stronger than that of 17S62, 5S54 was named *slac1* and was used in the analyses for the remainder of this study. It was confirmed that *slac1* plants of the M4 generation retained the low leaf temperature phenotype (Fig. 4).

Phenotype–genotype correlations in the M4 population indicated that all heterozygous plants had a phenotype indistinguishable from that of wild-type plants (Supplementary Table S2 available at *JXB* online), suggesting that *slac1* is a recessive mutation. It was confirmed that *SLAC1* transcripts accumulated in the *slac1* mutant normally (Fig. 3C), showing that the *slac1* mutation does not alter *SLAC1* functioning at the transcriptional level. Three amino acid substitutions occurred in the *slac1* mutant (Table 1), R268K, A356V, and G372K. The replacement of arginine with lysine in R268 is a conservative change and results in the substitution of a residue identical to that found in *Arabidopsis*, K255 (Fig. 2). Therefore, it is unlikely that R268K alters the function of *SLAC1*. A356 and G372 are conserved in *Arabidopsis* SLAC1 (Fig. 2), whereas the rice mutant contains substituted amino acids in these positions that have different properties, valine and lysine, respectively. In particular, G372 is known to be >95% identical within the plant subfamily SF1A for SLAC1 and within the TehA subfamily (Chen et al., 2010),
Increased leaf photosynthesis in a rice SLAC1-deficient mutant

and is possibly important for SLAC1 function. As reported in Arabidopsis, the rice SLAC1 channel was predicted to be composed of 10 helices of two layers (Chen et al., 2010; Du et al., 2011). In the SLAC1 homology model, A356 and G372 are on the middle portions of putative inner transmembrane segment 7 (TM7) and outer segment 8 (TM8), respectively (Supplementary Fig. S1 at JXB online). Spatially A356 is located in the centre of channel near the predicted gate of the channel F461. Therefore, A356V substitution would be expected to affect channel behaviour negatively.

Stomatal density

In many plants, stomatal density positively correlates with stomatal conductance \( (g_s) \) as well as with water use efficiency (WUE) (Hetherington and Woodward, 2003; Masle et al., 2005; Xu and Zhou, 2008; Liu et al., 2012). A low leaf temperature phenotype can result from increased stomatal density via water loss through enhanced transpiration. Stomatal density was therefore measured in wild-type and slac1 mutant plants. A comparison of stomatal density in the middle portion of the flag leaf revealed that the slac1 mutation did not cause a

Table 1. Mutant lines found for the SLAC1 gene region

| Line | Base change | Position from ATG | Amino acid change | Region          |
|------|-------------|-------------------|-------------------|-----------------|
| 15S28| G/A         | 37                | Gly/Ser           | CDS (exon 1)    |
| 17S62| C/T         | 493               | Pro/Ser           | CDS (exon 1)    |
| 25S66| G/A         | 576               | Arg/Cys           | Intron          |
| 5S54 | C/T         | 896               | Ala/Val           | CDS (exon 2)    |
| 5S54 | C/T         | 1161              | Gly/Arg           | CDS (exon 2)    |
| 5S54 | G/C         | 1208              | Pro/Ser           | CDS (exon 2)    |
| 18S59| C/T         | 1715              |                   | CDS (exon 3)    |

Base change: original nucleotide/mutated nucleotide.
Amino acid change: original amino acid/changed amino acid.
significant change in stomatal density in either the adaxial or abaxial epidermis (Fig. 5). Together with an earlier report showing that SLAC1 deficiency does not affect stomatal density in Arabidopsis (Vahisalu et al., 2008), SLAC1 is not likely to be involved in the regulation of stomatal density.

**Gas exchange**

In Arabidopsis, SLAC1 is essential for stomatal closure in response to CO$_2$, and SLAC1-deficient mutants showed constitutively higher stomatal conductance, directly due to larger stomatal apertures (Negi et al., 2008; Vahisalu et al., 2010). Therefore, the response of $g_s$ and $A$ to changes in the aerial [CO$_2$] in slac1 and wild-type plants was investigated by using a portable gas exchange fluorescence system. For the measurement, wild-type and slac1 plantlets were grown on soil under greenhouse conditions. Since the $g_s$ of field-grown rice is generally higher in the morning and around noon than in the afternoon (Shimono et al., 2010), measurements were taken at approximately noon (11:00–13:00h) on the middle portion of the sixth or seventh leaf of seedlings at the 7.5 stage (Itoh et al., 2005). Increases in [CO$_2$] from 350 ppm to 700 ppm induced a decrease in $g_s$ in the wild-type plants (Fig. 6A). In the slac1 mutant, stomatal conductance at 700 ppm was only slightly lower than that at 350 ppm and was maintained at a much higher level than in the wild-type plants. The means of hourly values in the mutant plants at 350 ppm and 700 ppm were ~150% and ~170% higher, respectively, than those in wild-type plants (Fig. 6B). During the same period, increases in [CO$_2$] resulted in an increase in the rate of photosynthesis ($A$) in both wild-type and mutant plants (Fig. 6A), but the value for the mutant remained significantly higher than for wild-type plants. The slac1 mutant showed ~50% and ~40% higher mean hourly values at 350 ppm and 700 ppm, respectively (Fig. 6B).

Since higher stomatal conductance may enhance CO$_2$ diffusion into chloroplasts, the photosynthetic activity of plants was assessed through CO$_2$ response curves. Analysing response curves between $A$ and leaf $C_i$ enables estimation of the relative contribution of stomatal versus non-stomatal (biochemical) limitations to photosynthesis (von Caemmerer and Farquhar, 1981). Compared with wild-type plants, mutant plants showed a slightly higher but similar $A$ that increased proportionally to the $C_i$ until saturation was reached (Fig. 7). On the other hand, the $C_i/C_a$ ratio in the slac1 mutant was significantly higher than that in wild-type plants. The slac1 mutant showed an ~50% and ~30% higher $C_i/C_a$ ratio at 800 ppm and 350 ppm $C_a$, respectively (Fig. 7). These observations suggest that the higher $A$ observed in the mutant leaves was due to the larger $g_s$.

Higher CO$_2$ environments accelerate carbon fixation and modulate carbon and nitrogen balance via changes in the levels of structural and non-structural carbohydrates and proteins (Allen et al., 1988). Since the slac1 mutant possessed a higher photosynthetic capacity compared with wild-type plants, the internal balance between carbon and nitrogen may be altered. Therefore, the effect of SLAC1 deficiency on the carbon and nitrogen contents of the same leaves used for $A/C_i$ measurement was examined. However, significant differences were not observed in either the carbon and nitrogen content or the C:N ratio between the wild-type and slac1 mutant leaves (Fig. 8).

**Discussion**

In this study the first data are provided showing that a homologue of the SLAC1 protein originally identified in Arabidopsis is also involved in stomatal regulation in rice. Under various conditions, there is a striking correlation between photosynthetic capacity and stomatal conductance in rice (Ishihara and Saito, 1987; Hirasawa et al., 1988); however, the relative importance of stomatal conductance in restricting the supply of CO$_2$ for metabolism (stomatal limitation) and in altering metabolism to decrease the potential rate of photosynthesis (non-stomatal limitation) is still unclear. A rice SLAC1-deficient mutant slac1 was developed and observed, and it was concluded that in rice SLAC1 is also involved in stomatal closure, and that stomatal conductance limits photosynthesis under well-watered growth conditions.

SLAC1 function is conserved between Arabidopsis and rice

A putative SLAC1 orthologue was initially designated based on protein sequence identity and intron/exon structure. These
Increased leaf photosynthesis in a rice SLAC1-deficient mutant

Features of the SLAC1 gene are similar between Arabidopsis and rice, and quite different from other SLAC1 homologues (Figs 1, 2; Table 1), as mentioned previously (Vahisalu et al., 2008). Alignment of predicted secondary structures (Supplementary Fig. S1 at JXB online) also indicated that this protein has structural features in common with slow anion channel proteins (Chen et al., 2010; Du et al., 2011). Accumulation of SLAC1 mRNA occurred in the early leaf developmental stage P4 along with that of other genes involved in stomatal development, STOMAGEN and HT1. Since other genes related to stomatal formation also occur during this stage (Liu et al., 2009), this suggests the involvement of SLAC1 in rice stomatal development. Compared with STOMAGEN and HT1, SLAC1 transcript preferentially accumulated in younger leaves (P0–P3 and early P4 stages). Conversely, in mature leaves, SLAC1 transcript accumulated only slightly. Considering that SLAC1 as well as HT1 regulates stomatal closure and HT1 transcripts accumulated greatly in mature P5 leaves, SLAC1 may be long-lived protein and turns over slowly.

The slac1 mutation caused an increase in stomatal conductance concomitant with lowered leaf temperatures, but did not affect other observable morphological phenotypes of the mutant plants. For example, stomatal density is a representative stomatal phenotype and is known to respond to various environmental factors, such as elevated [CO2] and drought stress (Woodward, 1987; Xu and Zhou, 2008), but stomatal density was not affected in the slac1 mutant (Fig. 5).

Stomatal limitations to photosynthesis in rice

Increases in stomatal conductance caused by SLAC1 deficiency resulted in enhanced rates of photosynthesis (Fig. 6). A comparison of the A–C curves between the mutant and wild-type seedlings (Fig. 7) suggested that stomatal closure is the main limiting factor for the photosynthetic rate in response to alterations in [CO2]. It is generally accepted that in photosynthesizing leaves, stomatal conductance is correlated with photosynthetic rate and coordinated with the CO2 requirement of the mesophyll, such
that the $C_i/C_a$ ratio is maintained at a constant value (Wong et al., 1979, 1985; Sharkey and Raschke, 1981). Guard cells are thought to sense $C_i$ rather than $C_a$, since ambient [CO$_2$] does not vary in nature; however, in the slac1 mutant, an artificial increase in stomatal conductance results in an increase in $C_i$ at constant $C_a$ that does not, however, seem to be recognized by mesophyll cells, as the $A$–$C_i$ curve is not significantly affected by the mutation (Figs 6, 7). This finding suggests that in rice the $C_i/C_a$ ratio is determined by the $C_i$ value maintained by the stomatal conductance, and that the final photosynthetic capacity seems to be determined by stomatal conductance.

Growth under constant environmental conditions in the greenhouse is, however, quite artificial. To avoid drought stress, rice plants used in this study were grown in growth chambers or in the greenhouse at 60–80% relative humidity. The relative humidity in the cuvette of a gas exchange fluorometer was also kept fairly high (60%). Preliminary observation suggested that after the slac1 mutant plants were transferred to low humidity conditions (<40%) from the standard condition, young leaves of slac1 seedlings began to roll or curl, probably caused by water loss. Similarly, the photosynthetic rate of mutant leaves significantly decreased and became quite unstable when the humidity in the cuvette was set below 50%. Therefore, the present data and conclusion are based on the assumption that, during plant growth, drought stress is avoided and that the water conditions are optimized for photosynthesis. In fact, in field conditions, the tiller number and plant height of the slac1 plants at the end of the tillering stage were lower than in the wild type (by ~5% and ~4%, respectively), suggesting that enhanced drought stress caused by increased stomatal opening exceeds the benefits of increased CO$_2$ assimilation.

The data indicate that under controlled environmental conditions where the biochemical capacity of the photosynthetic rate is not limited and drought stress is avoided, stomatal conductance can become the primary determinant of photosynthetic capacity in rice. An artificial increase in stomatal conductance via genetic engineering may, therefore, improve the productivity and yield of rice plants. The finding that modulation of a single gene, SLAC1, increased photosynthetic capacity provides a new tool for the further examination of stomatal engineering for photosynthetic adaptation of rice.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Alignment of the SLAC1 model structure and its template H. influenzae TehA.

Table S1 Primer sequences used in this study.

Table S2 Genotype–phenotype correlation in the slac1 mutant.

Acknowledgements

We are grateful to M. Nishi and R. Kaji for their technical assistance. Our appreciation is extended to Dr J. Negi and
Y. Yamamoto for fruitful discussion. This work was supported by the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN), a Grant-in-Aid for Scientific Research on Innovative Areas (No. 21114002), and the Ministry of Education, Science and Culture of Japan (no. 22570045).

References

Adachi S, Tsuru Y, Nito N, Murata K, Yamamoto T, Ebitani T, Ookawa T, Hirasawa T. 2011. Identification and characterization of genomic regions on chromosomes 4 and 8 that control the rate of photosynthesis in rice leaves. Journal of Experimental Botany 62, 1927–1938.

Allen LH, Yu JCV, Valle RR, Boote KJ, Jones PH. 1988. Nonstructural carbohydrates and nitrogen of soybean grown under low CO2 enrichment. Annals of Botany 54, 391–397.

Bai S, Jiang Y, Jia H, Li Y, Liu T, Ohashi-Ito K, Bergmann DC. 2009. Orthologs of Arabidopsis thaliana stomatal biHLH genes and regulation of stomatal development in grasses. Development 136, 2265–2276.

Chen YH, Hu L, Punta M, Bruni R, Hillerich B, Kloss B, Rost B, Love J, Siegelbaum SA, Hendrickson WA. 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nature Protocols 4, 363–371.

Hetherington AM, Woodward FI. 2003. The role of stomata in sensing and driving environmental change. Nature 424, 901–908.

Ishihara K, lida O, Hirasawa T, Ogura T. 1979. Relationship between nitrogen content in leaf blades and photosynthetic rate of rice plants with reference to stomatal aperture and conductance. Journal of Crop Science 48, 543–550.

Ishihara K, Saito K. 1987. Diurnal courses of photosynthesis, transpiration, and diffusive conductance in the single-leaf of the rice plants grown in the paddy field under submerged condition. Japanese Journal of Crop Science 56, 8–17.

Itoh J, Nonomura K, Ikeda K, Yamaki S, Inukai Y, Yamagishi H, Kitano H, Nagato Y. 2005. Rice plant development: from zygote to spikelet. Plant and Cell Physiology 46, 23–47.

Jain M, Nijhawan A, Tyagi AK, Khurana JP. 2006. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. Biochemical and Biophysical Research Communications 345, 646–651.

Kelley LA, Sternberg MJ. 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nature Protocols 4, 363–371.

Kusumi K, Chono Y, Gotoh E, Tsuyama M, Iba K. 2010a. Chloroplast biogenesis during the early stage of leaf development in rice. Plant Biotechnology 27, 85–90. http://dx.doi.org/10.5511/plantbiotechnology:27.85

Kusumi K, Hirotsuka S, Shimada H, Chono Y, Matsuda O, Iba K. 2010b. Contribution of chloroplast biogenesis to carbon–nitrogen balance during early leaf development in rice. Journal of Plant Research 123, 617–622.

Kusumi K, Sakata C, Nakamura T, Kawasaki S, Yoshimura A, Iba K. 2011. A plastid protein NUS1 is essential for build-up of the genetic system for early chloroplast development under cold stress conditions. The Plant Journal 68, 1039–1050.

Liu J, Zhang F, Zhou J, Chen F, Wang B, Xie X. 2012. Phytochrome B control of total leaf area and stomatal density affects drought tolerance in rice. Plant Molecular Biology 78, 289–300.

Liu T, Ohashi-Ito K, Bergmann DC. 2009. Orthologs of Arabidopsis thaliana stomata biHLH genes and regulation of stomatal development in grasses. Development 136, 2265–2276.

Masle J, Gilmore SR, Farquhar GD. 2005. The ERECTA gene regulates plant transpiration efficiency in Arabidopsis. Nature 436, 866–870.

Merlot S, Mustilli AC, Genty B, North H, Lefebvre V, Sotta B, Vavasseur A, Giraudat J. 2002. Use of infrared thermal imaging to isolate Arabidopsis mutants defective in stomatal regulation. The Plant Journal 30, 601–609.

Nemoto K, Yamazaki K. 1993. Correlative development of vegetative organs. In: Matsuoka T, Hoshikawa K, eds. Science of the rice plant. Vol. 1. Tokyo: Food and Agriculture Policy Research Center, 625–627.

Ohsumi A, Hamasaki A, Nakagawa Y, Oba Y, Takahashi H, Kawai-Yamada M, Uchimiya H, Hashimoto M, Iba K. 2008. CO2 regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. Nature 452, 483–486.

Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. 2004. UCSF Chimera—a visualization system for exploratory research and analysis. Journal of Computational Chemistry 25, 1605–1612.

Saji S, Bathula S, Kubo A, Tamaoki M, Kanna M, Aono M, Nakajima N, Nakaji T, Takeda T, Asayama M, Saji H. 2008. Disruption of a gene encoding C4-dicarboxylate transporter-like protein in Arabidopsis.

Disruption of a gene encoding C4-dicarboxylate transporter-like protein in Arabidopsis.
protein increases ozone sensitivity through deregulation of the stomatal response in Arabidopsis thaliana. Plant and Cell Physiology 49, 2–10.

Sharkey TD, Raschke K. 1981. Separation and measurement of direct and indirect effects of light on stomata. Plant Physiology 68, 33–40.

Shimono H, Okada M, Inoue M, Nakamura H, Kobayashi K, Hasegawa T. 2010. Diurnal and seasonal variations in stomatal conductance of rice at elevated atmospheric CO(2) under fully open-air conditions. Plant, Cell and Environment 33, 322–331.

Sugano SS, Shimada T, Imai Y, Okawa K, Tamai A, Mori M, Hara-Nishimura I. 2010. Stomagen positively regulates stomatal density in Arabidopsis. Nature 463, 241–244.

Suzuki T, Eiguchi M, Kumamaru T, Satoh H, Matsusaka H, Moriguchi K, Nagato Y, Kurata N. 2008. MNU-induced mutant pools and high performance TILLING enable finding of any gene mutation in rice. Molecular Genetics and Genomics 279, 213–223.

Taylaran RD, Adachi S, Ookawa T, Usuda H, Hirawata T. 2011. Hydraulic conductance as well as nitrogen accumulation plays a role in the higher rate of leaf photosynthesis of the most productive variety of rice in Japan. Journal of Experimental Botany 62, 4067–4077.

Till BJ, Reynolds SH, Greene EA, et al. 2003. Large-scale discovery of induced point mutations with high-throughput TILLING. Genome Research 13, 524–530.

Vahisalu T, Kollist H, Wang YF, et al. 2008. SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. Nature 452, 487–491.

Vahisalu T, Puzorova I, Brosche M, et al. 2010. Ozone-triggered rapid stomatal response involves the production of reactive oxygen species, and is controlled by SLAC1 and OST1. The Plant Journal 62, 442–453.

von Caemmerer S, Farquhar GD. 1981. Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. Planta 153, 376–387.

Willmer CM, Fricker MD. 1996. Stomata, 2nd edn. London: Chapman & Hall.

Wong SC, Cowan IR, Farquhar GD. 1979. Stomatal conductance correlates with photosynthetic capacity. Nature 282, 424–426.

Wong SC, Cowan IR, Farquhar GD. 1985. Leaf conductance in relation to rate of CO2 assimilation: I. Influence of nitrogen nutrition, phosphorus nutrition, photon flux density, and ambient partial pressure of CO2 during ontogeny. Plant Physiology 78, 821–825.

Woodward FI. 1987. Stomatal numbers are sensitive to increases in CO2 from pre-industrial levels. Nature 327, 617–618.

Xu Y-F, Ookawa T, Ishihara K. 1997. Analysis of the photosynthetic characteristics of the high-yielding rice cultivar Takanari. Japanese Journal of Crop Science 66, 616–623.

Xu Z, Zhou G. 2008. Responses of leaf stomatal density to water status and its relationship with photosynthesis in a grass. Journal of Experimental Botany 59, 3317–3325.