Leaf Photosynthesis and Its Genetic Improvement from the Perspective of Energy Flow and CO₂ Diffusion

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Abstract: Single-leaf photosynthesis is a fundamental process in plant biomass production, and is a major research topic in crop physiology. This paper reviews the recent achievements of research on the physiological determinants of the photosynthetic capacity from the perspective of energy flow and CO₂ diffusion. Measurement of chlorophyll fluorescence is a popular method to diagnose the function of photosystem II, and is useful to assess the susceptibility to photoinhibition and allocation of energy, which are keys to improving both stress resistance and photosynthetic productivity. Mesophyll conductance (gm) is the conductance to CO₂ diffusion from intercellular airspaces to the chloroplast, and was long thought to be determined by leaf anatomical properties. However, recent studies showed that environmental conditions affect gm. It is possible that gm is affected by the gating of the CO₂-permeable aquaporins (cooporins). Stomatal morphology is revealed to be an important factor affecting gas exchange both in crop plants and in Arabidopsis thaliana. The knowledge of the stomatal differentiation in Arabidopsis will be applicable to various crops. gm, stomatal conductance (gs) and leaf nitrogen content are the main factors to cause difference in leaf photosynthesis among rice lines, and recent activities are conducted to find genes to manipulate these factors. Although the association of leaf photosynthesis with crop productivity still has a large ‘missing link’, these achievements strongly suggest that the leaf photosynthetic capacity can be genetically improved in crop species.

Key words: Chlorophyll fluorescence, Cooporin, Leaf nitrogen content, Mesophyll conductance, Stomatal conductance, Stomatal density.

Single-leaf photosynthesis is a fundamental process in plant biomass production. The process of photosynthesis can be classified into three major steps: light harvesting and the subsequent conversion of light into chemical energy (NADPH and ATP) through photosystem II (PSII) and photosystem I (PSI) in the thylakoid membrane in chloroplasts; the biochemical process of the Calvin-Benson cycle in the stroma; and the supply of carbon dioxide from the atmosphere to the site of carboxylation. Thus, the net photosynthetic rate per unit leaf area (Pn) is determined through the combined action and interactions of these steps. Using a biochemical model, Farquhar et al. (1980) successfully described these processes, and this powerful model has been widely applied to study the mechanism of Pn determination in C₃ plants.

The impact of Pn on plant canopy photosynthesis was quantitatively predicted by Monsi and Saeki (1953), who indicated that canopy photosynthesis is determined by the
leaf area index, light extinction coefficient and \( P_n \). A higher \( P_n \) is suggested to be beneficial for crop production based on both field observations (Horie et al., 2006) and simulation modeling (Long et al., 2006). However, the actual link between \( P_n \) and biomass production has yet to be elucidated because the process of biomass production consists of a large number of interactions, including source-sink balances and the environmental responses of the plant. For instance, a negative relationship is occasionally observed between \( P_n \) and seed yield (Evans, 1993). Hence, the benefits of genetically improving \( P_n \) on biomass production and yield should be tested under field conditions. To develop plant materials showing an improved productivity, we need to understand the key traits involved in the underlying genetic variation and the environmental response of \( P_n \).

For decades, many technical advances have been greatly accelerating research on photosynthesis. Portable gas exchange systems, such as the LI-6400 system (Li-COR, USA), make it possible to measure instantaneous \( P_n \) even under field conditions. Using chlorophyll (Chl) fluorescence measurement systems with a pulse-amplitude-modulated (PAM) fluorometer, it is possible to quantify the degree of the stress of a leaf and estimate the electron transport rate of PSII (Maxwell and Johnson, 2000). Furthermore, to estimate the CO\(_2\) diffusion inside leaves during photosynthesis, the carbon isotope discrimination (\( \Delta \)) method has been developed (Farquhar et al., 1982). Simultaneous measurements of gas exchange and Chl fluorescence or \( \Delta \) can provide more detailed information, such as the alternative electron transport rate (Miyake and Yokota, 2000) and mesophyll conductance (\( g_m \)) to CO\(_2\) diffusion (Evans et al., 1986; Harley et al., 1992; Baker, 2008). A remarkable feature of this technology is that we can determine the internal condition of a single leaf through rapid and non-destructive measurements. The advantage of such high-throughput measurements of photosynthetic performance is maximized when combined with the use of various genetic materials and technologies. The identification of quantitative trait loci (QTLs) using advanced mapping populations, including chromosomal segment substitution lines (CSSLs), is expected to be a powerful approach for understanding the genetic factors underlying the natural variation of \( P_n \). Furthermore, progress in the field of molecular biology has made it easier to manipulate the function of targeted genes, not only in model plants but also in crop species.

A mini-symposium regarding the recent achievements of research on the physiological determinants of \( P_n \) in crops and other plant species was held at the 235th Conference of the Crop Science Society of Japan on 29th March, 2013. Special concern was paid to the energy flow and CO\(_2\) diffusion in the photosynthetic system. This review summarizes our concern based on the discussion at the mini-symposium. First, assessments of photosynthesis-related parameters in rice cultivars using Chl fluorescence-based techniques are described in reference to low-nitrogen (N) availability. Second, the significance and the environmental response of \( g_m \) to CO\(_2\) diffusion are discussed, along with the technical issues involved in the estimation of \( g_m \) using the \( \Delta \) method. Third, stomatal density is highlighted as an important factor affecting gas exchange in the leaf, and the physiological consequences of the genetic manipulation of stomatal density are reviewed. Lastly, physiological factors associated with the natural variation of \( P_n \) in rice varieties are discussed, and genetic enhancement of \( P_n \) is described based on QTL analysis of leaf photosynthesis. Herein, we present the various perspectives regarding research on photosynthesis and their future potential contributions to crop science.

1. **Assessment of susceptibility to photoinhibition and the allocation of absorbed light energy in PSII in rice leaves based on Chl fluorescence measurements**

Thus far, most crop scientists aiming at the genetic improvement of leaf photosynthesis in crop plants have mainly focused on \( P_n \) and the content and activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which is the key enzyme involved in the Calvin-Benson cycle (e.g., Evans and Seemann, 1984; Hubbart et al., 2007). Recently, it has become possible to easily diagnose the function of PSII in the leaf using PAM fluorometers. The physiological factors responsible for the differences in the productivity of rice cultivars under low-N input conditions using PAM-Chl fluorescence measurements have been reported (Kumagai et al., 2007, 2009, 2010, 2012). This section describes the physiological factors associated with photoinhibition and related processes under low-N conditions and how modification of these processes may be a useful strategy for increasing leaf photosynthesis.

Although performing Chl fluorescence measurements is simple, the underlying theoretical aspects and interpretation of these data are complex. Some excellent reviews have discussed the theoretical background of these measurements and their analysis (e.g., Maxwell and Johnson, 2000). The light energy absorbed by the PSII antennae in the leaves of plants can either be utilized via photochemistry or dissipated via thermal processes and fluorescence. The fate of absorbed light energy and the degree of photoinhibition of PSII in the leaves can be assessed based on Chl fluorescence measurements. The degree of photoinhibition of PSII can be estimated from the ratio of variable Chl fluorescence to maximal fluorescence (\( F_v/F_m \)). Photoinhibition is defined as the light-induced loss of photosynthetic activity. Absorbed light energy may be defined as excessive when it exceeds the photosynthetic capacity to use it. It is believed that
excessive light energy produces reactive oxygen species (ROS; e.g., $^1$O$_2$, O$_2^-$, H$_2$O$_2$), which can cause photoinhibition of PSII (Foyer and Noctor, 1999). The extent of photoinhibition of PSII depends not only on the rate of D1 protein degradation but also on the rate of D1 protein synthesis within chloroplasts (Kyle et al., 1984). A number of hypotheses have been put forward to explain the molecular mechanism underlying the photoinhibition of PSII (Keren and Krieger-Liszkay, 2011). Quantifying the fate of light energy absorbed by PSII is significantly important for studying the responses of photosynthesis and photoinhibition to environmental stresses. The ‘puddle’ and ‘lake’ models of energy allocation were proposed by Demmig-Adams et al. (1996) and Hendrickson et al. (2004), respectively. In the puddle model, PSII centers are hypothesized to exist solely within the energy-processing systems. This model can be divided into three categories: PSII photochemistry, Dissipation and Excess. In the lake model, all PSII centers are instead hypothesized to be coupled with each other to exchange energy. This model can be used to categorize energy fluxes via linear PSII electron transport ($J_{\text{psII}}$), light-dependent thermal dissipation ($J_{\text{NPQ}}$) and fluorescence and light-independent basal thermal dissipation ($J_{\text{TD}}$). Moreover, simultaneous measurements of photosynthetic gas exchange and Chl fluorescence can be used to estimate the allocation of the total light energy absorbed by PSII to the various processes of $J_{\text{psII}}$ (photosynthesis, $J_c$; photorespiration, $J_o$; alternative electron transport, $J_a$) (Miyake and Yokota, 2000).

The effects of N deficiency on photosynthesis, photoinhibition and the allocation of light energy absorbed by PSII in flag leaves using two rice cultivars, Shirobeniya and Akenohoshi have been studied (Kumagai et al., 2010). The grain yield and $P_n$ in flag leaves in Akenohoshi were found to be superior to those in Shirobeniya under low-N conditions (Kumagai et al., 2009, 2012). Energy allocation was estimated based on the lake model and measured under high irradiance (1,500 μmol m$^{-2}$ s$^{-1}$ PPFD). In the low-N plants of the two cultivars, $J_{\text{NPQ}}$ accounted for 46 – 49% of the total light absorbed by the PSII antenna, whereas in the standard-N plants, this percentage was 42% (Fig. 1). In contrast, there was no difference in the fraction of $J_{\text{TD}}$ between the two N conditions, with a value of 21% being found for both. When grown under low-N conditions, the fractions of light absorbed by PSII and used for $J_o$, $J_c$ and $J_a$ were 17 – 18, 9 and 4 – 6%, respectively, whereas under standard-N condition, the fractions used for $J_o$, $J_c$ and $J_a$ were 21 – 23%, 13 – 14% and 2%, respectively. Most of $J_o$ is thought to account for the electron flux to the water-water cycle. This result suggested that both xanthophyll cycle-dependent thermal dissipation and the water-water cycle were up-regulated under the low-N condition. The water-water cycle is unavoidably coupled with the generation of ROS (Asada, 1999), which potentially cause photooxidative damage to thylakoid membrane and stroma proteins. The limiting step of the water-water cycle is the photoreduction of O$_2$ to O$_2^-$, and both superoxide dismutase (SOD) and ascorbate peroxidase (APX) are key enzymes involved in the process of ROS scavenging (Endo and Asada, 2006). It was found that in the two cultivars, the increased $J_o$ was accompanied by enhanced activity of SOD under the low-N condition. However, APX activity in Akenohoshi was constant, regardless of the N level, whereas the activity in Shirobeniya was decreased significantly under the low-N condition. Additionally, the low-N supply resulted in a more significant increase of susceptibility to photoinhibition (reduction of $F_v/F_m$ after high irradiance), H$_2$O$_2$ accumulation and lipid peroxidation within leaves in Shirobeniya than in Akenohoshi. The higher H$_2$O$_2$ levels in the low-N Shirobeniya plants could be explained by lower APX activity. H$_2$O$_2$ inhibits the synthesis of the D1 protein.
(Takahashi and Murata, 2008). These results indicated that the increased susceptibility to photoinhibition observed in the low-N plants of the Shirobeniya cultivar was mainly due to oxidative damage to the chloroplasts, resulting from a decreased $\text{H}_2\text{O}_2$-scavenging capacity. It was therefore concluded that the $\text{H}_2\text{O}_2$-scavenging capacity could be an important factor in determining the difference between the cultivars regarding rice production under the low-N condition.

As described above, the combined use of Chl fluorescence and gas exchange measurements could be effective for evaluating the response of energy allocation and photoinhibition to environmental stresses; however, there are major problems that should be considered. When Chl fluorescence measurements are carried out using conventional PAM fluorometers, the observed Chl fluorescence represents a mixture of signals emitted from the mesophyll cell layers facing the detector because the measuring light cannot penetrate deeply within a leaf (Bornman et al., 1991; Oguchi et al., 2011). Because the degree of photoinhibition is light dependent, the intra-leaf light gradient creates a gradient of photoinhibition within a leaf (Schreiber et al., 1996). $J_s$ is estimated by subtracting $J_i$ and $J_e$ (estimated using the gas exchange method) from $J_{rsi}$ (estimated from Chl fluorescence), which could originate from different cell populations. Therefore, both $F_0/F_m$ and $J_e$ estimated by conventional techniques could be inaccurate. $J_i$ involves several pathways other than the water-water cycle, such as the cyclic electron flow around PSII and PSI, the mitochondrial alternative oxidase pathway and the pathways for mineral nutrient assimilation (Miyake, 2010). Methodologies should be established to assess the extent of photoinhibition and electron transport rates in whole leaves.

The maximum photosynthetic potential is rarely observed even under favorable conditions (Murchie et al., 1999). There are many potential reasons, but photoinhibition may be a major source of photosynthetic losses (Horton, 2000). The increased susceptibility to photoinhibition observed in stress-sensitive rice cultivars compared with stress-tolerant ones has been reported to be associated with a lower ROS-scavenging activity under various stress conditions, such as salt stress (Vaidyanathan et al., 2003) and low temperature (Bonnecarrère et al., 2011), which is in good agreement with our results. Therefore, manipulation of ROS-scavenging capacities is highlighted as a target for the improvement of both photosynthetic productivity and stress resistance in crop plants. Several studies in rice have also suggested that japonica cultivars show more tolerance to photoinhibition than indica cultivars (Jiao and Ji, 2001; Jiao et al., 2003). More recently, Kasajima et al. (2011) reported that non-photochemical quenching (light-dependent thermal dissipation capacity) of japonica is higher than that of indica based on an analysis of a rice core collection. However, information about the genetic diversity in the susceptibility to photoinhibition and its physiological determinants remain limited. Chl fluorescence-imaging systems that can image related parameters in a large area have been recently developed (Baker and Rosenqvist, 2004). It is expected that this type of tool will promote the development of research on both the photosynthetic productivity and stress resistance of crop plants.

2. Effects of $\text{CO}_2$, irradiance and stomatal gating on mesophyll conductance

In C₃ leaves, the diffusion of $\text{CO}_2$ into the leaves is restricted by the stomata, and subsequent diffusion into chloroplasts is restricted by the intercellular airspaces and liquid phase (Fig. 2). The conductance to $\text{CO}_2$ diffusion from intercellular airspaces to the chloroplast is termed mesophyll conductance ($g_m$). $g_m$ has long been thought to be determined by leaf anatomical properties, e.g., the thickness of the mesophyll cell wall and/or the chloroplast surface area adjacent to the intercellular airspaces. However, it was recently found that aquaporin 1 (AQP1), which is located in the plasma membrane and inner envelope of chloroplasts (Uehlein et al., 2008), is permeable to $\text{CO}_2$ (Uehlein et al., 2012). Thus, it is possible that $g_m$ is affected by the gating of the $\text{CO}_2$-permeable aquaporins, which is called ‘cooporins’ to highlight $\text{CO}_2$-porins that are co-operating with other photosynthetic components such as carbonic anhydrase (Terashima et al., 2006). Here, to reconsider our understanding of $g_m$ based on recent studies, we review the effects of $\text{CO}_2$, irradiance and stomatal conductance ($g_s$) on the values of $g_m$.

Developments in tunable-diode laser absorption spectroscopy (TDLAS) have improved our ability to perform rapid measurements of $g_s$ using the carbon isotope discrimination ($\Delta$) method (Evans et al., 1986). Tazoe et al. (2011) examined the effects of $\text{CO}_2$ and $\text{O}_2$
concentrations on $g_s$ in tobacco ($Nicotiana~tabacum$), Arabidopsis ($Arabidopsis~thaliana$) and wheat ($Triticum~aestivum$) leaves by combining gas exchange with $\Delta$ measurements using TDLAS. At first, photosynthetic rate was measured for approximately 40 min at irradiance of 1500 $\mu$mol quanta m$^{-2}$ s$^{-1}$, inlet CO$_2$ of 400 ppm, and 21% O$_2$. Then, O$_2$ was changed to 2% O$_2$ to reduce the carbon isotope fractionation associated with photorespiration, and CO$_2$ concentration was changed to 200 ppm. After 30 min, when the CO$_2$ concentration was switched from 200 to 1000 ppm, $P_s$ was observed to rapidly increase in tobacco. $g_s$ gradually increased at 200 ppm CO$_2$, then gradually decreased following the shift to 1000 ppm. On the other hand, $g_s$ rapidly decreased when CO$_2$ was increased from 200 to 1000 ppm in 2% O$_2$. The rapid change of CO$_2$ levels from 200 to 1000 ppm under 2% O$_2$ decreased $g_s$ by 40% in tobacco, 26% in Arabidopsis and 36% in wheat. Thus, $g_s$ decreased with increasing CO$_2$ in 2% O$_2$, similar to the response of $g_s$, whereas the response speed of $g_s$ was faster than that of $g_s$. The variation in $g_s$ may be caused by the gating of cooporins, but the effects of the CO$_2$ concentration on the cooporins are still unknown.

In tobacco and Arabidopsis, the response to CO$_2$ was also measured in 21% O$_2$ (Tazoe et al., 2011). At low CO$_2$ concentrations, $P_s$ was found to be greater under 2% than 21% O$_2$, as expected due to the suppression of photorespiration in 2% O$_2$. Although $g_s$ showed a similar increase with decreasing CO$_2$ in 21% O$_2$, $g_s$ was independent of CO$_2$ levels and did not increase, even at 200 ppm. The difference in the CO$_2$ response of $g_s$ between 2% and 21% O$_2$ can be partially explained by the effects of photorespiration because under low CO$_2$, $g_s$ varies greatly in 21% O$_2$ depending on the fractionation values during photorespiration in the $\Delta$ model (Tazoe et al., 2011). Furthermore, the refixation of respired CO$_2$ would also affect the estimates of $g_s$. Busch et al. (2013) indicated that in rice ($Oryza~sativa$) and wheat leaves, chloroplasts and their extrusions cover nearly the whole mesophyll surface, and 24–38% of photosynthesized and respired CO$_2$ is refixed within the cells. This effect of CO$_2$ refixation on estimates of $g_s$ has recently been reassessed (Tholen et al., 2012) but it is still under debate.

The response of $g_s$ to irradiance has been studied using various methods (von Caemmerer and Evans, 1991; Flechas et al., 2007), but there is still controversy regarding whether the $g_s$ observed at very low irradiance is a computation artifact. For example, under the ordinary $\Delta$ method, the magnitude of fractionation associated with photorespiration and day respiration can greatly affect the estimated $g_s$ when $P_s$ is small (Tazoe et al., 2009). To reduce the effects of respiration on these calculations, Tazoe et al. (2009) estimated the response of $g_s$ to irradiance in wheat leaves using a dataset in which $P_s$ was over 10 $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$ under 2% O$_2$. Consequently, between 200 and 1500 $\mu$mol quanta m$^{-2}$ s$^{-1}$, $g_s$ was independent of irradiance (Tazoe et al., 2009). On the other hand, Douthe et al. (2012) reported that $g_s$ in three $Eucalyptus$ species declined when irradiance decreased from 600 to 200 $\mu$mol quanta m$^{-2}$ s$^{-1}$. They recalculated $g_s$ using various fractionation values during both day respiration and photorespiration in the $\Delta$ model, but the $g_s$ estimated with several different values was still responsive to irradiance. If the observed response of $g_s$ to irradiance is not an artifact due to uncertainties in parameters related to respiration, $g_s$ may be regulated by the gating of aquaporins responding to irradiance as well as CO$_2$.

When the effect of various environmental factors on $g_s$ is investigated, there is concern about the involvement of $g_s$. In earlier studies, $g_s$ was shown to be positively correlated with $g_s$ (Centritto et al., 2003; Warren, 2008a). To investigate the effect of $g_s$ on $g_s$, Tazoe et al. (2011) measured the response of $g_s$ to CO$_2$ concentration using an Arabidopsis mutant ($ost1:~open~stomata~1$) that is insensitive to abscisic acid and whose stomata do not close at high CO$_2$ and in the dark (Merlot et al., 2002; Mustilli et al., 2002). In the $ost1$ mutant, $g_s$ was found to be higher than in wild type (WT) plants across all tested CO$_2$ concentrations, whereas $g_s$ in $ost1$ was identical to that in WT plants (Tazoe et al., 2011). Thus, $g_s$ can vary independently of $g_s$, but this independency would be observed only when $g_s$ is sufficiently high. Tholen et al. (2012) showed computationally that $g_s$ declined substantially when $g_s$ dropped below 0.1 mol m$^{-2}$ s$^{-1}$ but was stable when $g_s$ was greater than 0.1 mol m$^{-2}$ s$^{-1}$, assuming that chloroplast resistance accounted for more than 50% of mesophyll resistance and photosynthetic capacity did not vary with $g_s$. Thus, the decline of $g_s$ could lower $g_s$ in the calculation model, but it is difficult to demonstrate this experimentally using the $\Delta$ method because of difficulties in measuring $\Delta$ and estimating $g_s$ since stomata are closing.

$g_s$ has been considered to be a second limitation factor for CO$_2$ diffusion, following the stomata. The basic research on $g_s$ is steadily advancing, and the presence of CO$_2$-permeable aquaporins was recently confirmed in Arabidopsis (Uehlein et al., 2012). In the near future, it will become clear how cooporins modulate $g_s$ and whether the gating of the cooporins changes with irradiance and CO$_2$. Next, we have to consider how knowledge about $g_s$ can be applied to crop science. In recent years, to develop more productive crop species, a worldwide project aimed at introducing CO$_2$-concentrating mechanisms (CCMs) found in C$_4$ plants or cyanobacteria into C$_3$ plants, e.g., the C$_4$ rice project (Kajala et al., 2011; Meyer and Griffiths, 2013; Price et al., 2013) has been undertaken. However, to operate CCMs efficiently, a certain type of barrier to CO$_2$ is necessary to reduce CO$_2$ leakage from the CCM. For example, C$_4$ leaves develop Kranz anatomy and suberized cell walls in the bundle sheath cells, which are thought to
block CO₂ leakage. Cyanobacteria develop a carboxysome assembly in which bicarbonate is converted to CO₂ and fixed by Rubisco, which contributes to reducing the leakage of CO₂. If gs can be manipulated, it will contribute to the reduction of CO₂ leakage and improvement of the efficiency of photosynthesis in the C₄ rice.

3. Perspectives on the control of photosynthesis through manipulating the stomatal density

\( P_n \) is determined by the supply of CO₂ into the chloroplasts in the leaf and the carboxylation activity itself (Farquhar and Sharkey, 1982). Gas exchange through stomata is one of the most important processes involved in leaf photosynthesis because it greatly affects the diffusion of CO₂. The value of \( g_s \) changes dramatically in response to environmental factors such as light intensity, humidity, the water status of the plant and CO₂ concentrations (Hall and Kaufmann, 1975; Asamaa and Sober, 2012). These general responses are well explained by the optimization of CO₂ acquisition with the cost of transpiration. Additionally, genetic differences associated with \( g_s \) under optimum conditions and their impacts on \( P_n \) have been reported in crop plants (Ohsumi et al., 2007a; Lu et al., 1998). However, it is relatively unclear how such genetic differences related to \( g_s \) occur. Here, we describe the significance of the anatomy of leaf epidermis, particular of stomatal density, on gas exchange, and the genetic improvement of \( g_s \) and \( P_n \) in the model plant Arabidopsis.

Gas diffusion through the stomata is a physical process and can be described by stomatal density, longitudinal size, aperture and depth of stomata (Parlange and Waggoner, 1970; Franks and Beerling, 2009). Briefly, a greater stomatal density and a larger size of the stomata are expected to increase \( g_s \). Ohsumi et al. (2007b) reported a wide range of stomatal densities among various rice genotypes. Similarly, Ciha and Brun (1975) observed genotypic differences in stomatal density in soybean cultivars. The linkage between these variations and \( g_s \) has yet to be elucidated. Tanaka et al. (2010) observed over 70 genotypes of soybeans and found that the stomatal density varied from 192 mm⁻² to 332 mm⁻² (Fig. 3). The genotypic differences related to stomatal density were stable in field experiments for 2 yr. This finding showed that stomatal density is strongly regulated by genetic factors. Moreover, the maximum \( g_s \) estimated based on anatomical observations was significantly correlated with the actual stomatal conductance measured in the field. Interestingly, soybeans developed in the United States (US) had a higher stomatal density, of 23% on the average, compared with those developed in Japan. Overall, stomatal density has been suggested to have a significant effect on \( P_{\text{av}} \), implying that US soybean cultivars display an advantage regarding the efficiency of gas exchange and a higher \( P_n \).

The reason for such a clear regional bias in stomatal density is not clear. One candidate explanation is that the greater stomatal density is related to greater biomass productivity through a higher \( P_n \) in US cultivars. The soybean production in the US accounts for one-third of the world production (ERS/USDA, 2010), which is largely supported by intense breeding for a high yield performance. Kawasaki et al. (2010) have examined the yields of representative Japanese and US cultivars. The US cultivars had a higher yield than the Japanese cultivars on average across 2 locational and 2 yr experiments. It is likely that the applied selection pressure aimed at higher productivity resulted in the concomitant selection of genotypes with a higher stomatal density. Thus, genetic manipulation of stomatal density is an attractive strategy for improving the photosynthetic capacity.

The model plant Arabidopsis thaliana (L.) provides an ideal tool for evaluating the impact of genetic manipulation of stomatal density because the mechanism of stomatal development is well understood in this species (Bergmann and Sack, 2007). Briefly, signal peptides known as epidermal patterning factors (EPFs) secreted from mature stomata bind to receptors on immature pavement cells (Hara et al., 2007, 2009). This binding blocks the differentiation of the immature cells into new stomata. In addition to this ‘negative feedback’ regulation, Sugano et al. (2010) described a novel signal peptide referred to as STOMAGEN. STOMAGEN is a small, cysteine-rich peptide secreted from mesophyll tissues that positively regulates stomatal density by working antagonistically against EPFs. Overexpression or knockdown of the STOMAGEN gene (At4g12970) resulted in a drastic increase or decrease of stomatal density in Arabidopsis, respectively. Tanaka et al. (2013) applied these materials as an ideal tool to evaluate the physiological
consequence of the genetic manipulation of stomatal density.

**STOMAGEN** overexpressing (ST-OX) and knockdown (ST-RNAi) plants were grown together with a WT control. The stomatal density in the topmost leaf of 9-wk-old plants varied from 92 mm\(^{-2}\) in ST-RNAi to 699 mm\(^{-2}\) in ST-OX plants, while that of WT plants was 167 mm\(^{-2}\). The approximately 4 times greater stomatal density found in ST-OX led to a 2 times greater \(g_s\) compared with WT. Moreover, ST-OX showed a 30% higher \(P_n\) than WT. The enhancement of \(P_n\) observed in ST-OX was clear at a photosynthetic photon flux density higher than 300 \(\mu\text{mol quanta m}^{-2}\text{s}^{-1}\). The \(P_n\)-\(C_i\) curve, which represents the relationship between \(P_n\) and the intracellular concentration of CO\(_2\), did not vary significantly among the tested plants. Taking all of these results together, it was concluded that the higher \(P_n\) observed in ST-OX plants was mainly achieved via efficient gas diffusion due to the larger number of stomata in Arabidopsis.

Enhancement of \(P_n\) by increasing the stomatal density may be achievable not only in Arabidopsis but also in crop plants because gas diffusion itself is a physical process. The remaining issues are how to develop crop plants with a genetically increased stomatal density and what happens to such crop plants under various field conditions. Regarding the first issue, applying forward genetics methods, such as QTL analysis, can be a good strategy because stomatal density is easier to measure in a large plant population compared with other photosynthetic traits. Laza et al. (2010) reported QTLs related to stomatal density and size in rice, but the additive effect of each QTL was limited. According to their results, the natural variation of stomatal density might be regulated by large numbers of QTLs with small effects. Reverse genetics, which is an approach for evaluating the function of specific genes directly by manipulating DNA sequences, has been proposed as another strategy for this purpose. Recently developed screening systems based on this strategy, such as TILLING (Till et al., 2003; Anai, 2012), make it possible to effectively obtain plant materials with a mutation in a targeted gene in crop plants. Interestingly, genes homologous to EPFs and STOMAGEN from Arabidopsis were found with a high similarity in crop plants such as rice and soybean. This suggests that the mechanism of stomatal development is conserved among these species. Plant materials with a stomatal density manipulated over the range of natural variation can be obtained by mutagenizing these candidate genes related to stomatal development in crop species.

An important issue is what happens in crop plants with a manipulated stomatal density under various field conditions. Yoo et al. (2010) reported that a decreased stomatal density in an Arabidopsis mutant resulted in superior drought resistance. ST-RNAi plants were found to survive longer than WT plants under drought (Tanaka et al., 2013). Taken together, decreasing the stomatal density in crop plants may be an attractive strategy for improving water use efficiency (WUE) and drought resistance. On the other hand, enhanced gas exchange concomitantly causes increased transpiration and a decreased WUE. Although there was no significant difference in WUE between ST-OX and WT Arabidopsis plants in the experiments, sensitivity to drought is a potential risk of the strategy of increasing stomatal density in crop plants grown under various field environments. Optimization of the stomatal density will be important to overcome this problem following careful evaluations of the performance of crops with an increased stomatal density. It will also be useful to combine enhanced gas exchange with the improvement of mesophyll activity, including \(g_s\) or Rubisco activity. Such a ‘pyramiding’ of the improvement of photosynthetic components is expected to maximize the impact of the manipulation of stomatal density.

4. **Physiological and genetic factors underlying the natural variation of photosynthesis in rice**

The improvement of photosynthesis in individual leaves within the canopy is expected to increase the yield potential of rice, which is one of the most important crops in Asia (Hubbart et al., 2007). Although wide variations in \(P_n\) among rice cultivars have been shown in a number of studies (e.g., Murata, 1961; Kanemura et al., 2007), breeding programs aimed at increasing rice photosynthesis by utilizing these genetic resources have yet to be implemented (Flood et al., 2011). The rate of photosynthesis is affected by a number of physiological factors, including Rubisco content and CO\(_2\) diffusion from the atmosphere to the chloroplasts, which might be regulated by many genes. Therefore, to use these resources for breeding, we should elucidate the physiological factors that determine the observed varietal differences in \(P_n\) and identify the QTLs that control them (Flood et al., 2011). This section presents the physiological and genetic bases underlying the variation in \(P_n\) found in some rice lines that have been examined by Adachi et al. (2011a, 2011b, 2011c, 2013) and Taylaran et al. (2011) and the prospects for future improvement of rice photosynthesis.

The high-yielding *indica* cultivar Takanari displays the highest rate of light-saturating photosynthesis at an atmospheric CO\(_2\) concentration among rice cultivars (Kanemura et al., 2007; Hirasawa et al., 2010). We compared the physiological factors responsible for the high \(P_n\) found in Takanari with those in Koshihikari, the most popular cultivar in Japan, which exhibits a lower \(P_n\) (Taylaran et al., 2011). It is well known that \(P_n\) is affected by the leaf N content (LNC) because large amounts of N are invested in Rubisco, the primary CO\(_2\) fixation enzyme (Cook and Evans, 1983; Makino et al., 1984). The LNC of Takanari was found to be significantly higher than that of
Koshihikari when both cultivars received the same fertilizer regime. This higher LNC is expected to be one reason for the high $P_n$ found in Takanari. The high LNC of Takanari resulted not from the proportion of N distributed to the leaves, but from high plant N accumulation.

Because LNC affects not only Rubisco content but also many other photosynthesis regulators, including $g_s$, (Ishihara et al., 1979), it is important to examine $P_n$ of different varieties with the same LNC. When the LNC of Koshihikari was raised to the same level as that of Takanari through the application of additional N, both the $g_s$ and $P_n$ of Takanari remained higher than those of Koshihikari, but there was no difference in the response of $P_n$ to the intercellular CO$_2$ concentration between the two cultivars (Taylaran et al., 2011). These results indicate that the higher $P_n$ of Takanari is due to the higher $g_s$ even under the same LNC.

The response of leaf stomata to reduce leaf water potential in rice is much more sensitive compared to many other crops (Hirasawa, 1999). In fact, at a vapor pressure deficit of as small as 1.5 MPa, the value of $g_s$ in Koshihikari decreased due to the reduction of the leaf water potential. In contrast, the higher $g_s$ found in Takanari is supported by the maintenance of a higher leaf water potential through higher hydraulic conductance. This higher hydraulic conductance was due to the significantly greater root surface area. These results indicate that an increased root mass is important for increasing the $g_s$ in rice. The accumulation of N in plants is affected not only by their N assimilation capacity but also by the transportation of N to shoots via the transpirational stream (Cernusak et al., 2009). Plants with larger root systems exhibit high transpirational uptake of water (Hirasawa et al., 1992). Based on these results, the greater root mass found in Takanari might contribute to the higher N accumulation observed. The enhancement of root mass may be important not only for increasing $g_s$ but also for enhancing the accumulation of N in rice.

The diffusion of CO$_2$ from the atmosphere to the chloroplasts is regulated not only by $g_s$ but also by $g_m$, affects diffusion from the intercellular airspace to the chloroplasts and has been recognized as an important regulator of $P_n$ in recent years (Makino, 2011). Despite the wide variation of $g_s$ among species (see Warren, 2008b; Flexas et al., 2012), studies comparing $g_m$ among rice cultivars have not been reported. Among backcrossed inbreed lines derived from a cross between Koshihikari and Takanari, we found two high-photosynthesis lines, HP-a and HP-b, that showed a much higher $P_n$ than Takanari, approaching to that in maize, a C$_4$ plant (Adachi et al., 2013). However, the differences in LNC and $g_s$ between the HP lines and Takanari were small. Based on the results showing that the $g_m$ was significantly higher in the HP lines than in Takanari and that the relationship between the $P_n$ and CO$_2$ concentration in the chloroplasts in these lines was similar to that in Takanari when the LNC was similar, we concluded that the higher $P_n$ found in the HP lines was due mainly to the higher $g_m$ compared to Takanari. This conclusion indicates that the $P_n$ of rice can be greatly increased to the same level as found in maize if the rice exhibits greater $g_m$ than Takanari, in combination with high LNC and $g_s$ as found in Takanari. Mesophyll conductance may be a key factor for further increasing rice photosynthesis, and the variations of $g_m$ in rice cultivars and lines should therefore be examined well.

The size of $g_m$ is proportional to the cumulative surface area of the chloroplasts exposed to the intercellular airspace divided by the leaf surface area ($S_c/S$) among plants in the same functional group (Terashima et al., 2011). The HP lines display thick layers of mesophyll, like Takanari, but smaller mesophyll cells with more developed lobes than Takanari. These characteristics enlarge the cumulative mesophyll surface area and, thus, increase $S_c/S$. The smaller, more lobate mesophyll cells seem to be inherited from Koshihikari. Chonan (1967) referred to rice mesophyll cells as ‘armed’ parenchyma cells, and he speculated that their shape might enhance CO$_2$ diffusion inside the leaf. Our results support his speculation, although the greater number of mesophyll cells per leaf area is more critical for the increased $S_c/S$ observed in the HP lines. $g_m$ is controlled by several traits, including cell wall thickness, carbonic anhydrase activity and aquaporin activity, as well as $S_c/S$ (Hanba et al., 2004; Evans et al., 2009; Terashima et al., 2011). Further studies are necessary to clarify the factors that determine the differences in $g_m$ among rice cultivars and lines.

As illustrated above, we found that the differences in $P_n$ observed among rice lines can be explained by differences in LNC, $g_s$ and $g_m$ and we strongly suggest that simultaneously increasing these parameters has the potential to greatly improve rice photosynthesis (Fig. 4). An increased root mass and alteration of mesophyll anatomy are expected to be key factors for improving these.
parameters and, thus, improving $P_n$. Although the $P_n$ found in Koshihikari is much lower than that of Takanari, the HP progeny showed a much higher $P_n$ as a result of the cumulative effects of several key characteristics. This improvement suggests that photosynthesis can be significantly increased in rice through the use of already available genetic resources in appropriate combinations.

We have just begun QTL analysis using populations derived from a cross between Koshihikari and Takanari. Genetic analysis of populations derived from a cross between Koshihikari and the indica cultivar Habataki, which shows comparable $P_n$ to Takanari, allowed the mapping four QTL regions associated with $P_n$ on chromosomes 4, 5, 8 and 11 (Adachi et al., 2011a, 2011b). The Habataki segments of chromosome 5 and 11 were found to be responsible for increasing LNC, that of chromosome 8 for increasing $g$, and that of chromosome 4 for both traits. Pyramiding of the regions of chromosomes 4 and 8 in the Koshihikari genetic background increased $P_n$ to the same level as found in Habataki (Adachi et al., 2011c). Therefore, the mapping and pyramiding of QTLs for leaf photosynthesis may be useful strategies to improve leaf photosynthesis in rice.

Several QTLs related to leaf photosynthesis in rice have been reported (Teng et al., 2004; Hu et al., 2009; Takai et al., 2010; Gu et al., 2011), but only one gene underlying these QTLs has been identified thus far (Takai et al., 2013). We are working to identify the genes underlying these QTLs and to clarify the molecular mechanisms involved in the increase of $P_n$ by these genes. Genes associated with small and lobate mesophyll cell shape are targets to be identified in future research. Appropriate combinations of these genes are expected to increase $P_n$ to an unprecedented level. It will also be important to find a variety of alleles from diverse rice accessions. For this purpose, we need to develop multiple sets of populations, including CSSLs, using diverse accessions as donors for QTL mapping (Fukuoka et al., 2010) and rapid, high-accuracy methods to measure $P_n$ and traits related to photosynthesis (Flood et al., 2011). The close collaboration of crop breeders and crop scientists will promote breeding aimed at improving leaf photosynthesis in rice.

5. Conclusions and perspectives

In this review, we focused on the stability and capacity of single-leaf photosynthetic rate. It is possible to observe the flow of the energy or CO$_2$ in mesophyll tissues in detail by applying technical advances, including gas exchange measurement systems, Chl fluorescence measurement and carbon isotope discrimination. The progress in the field of molecular biology is making it easier to manipulate targeted traits involved in leaf photosynthesis. These achievements strongly suggest that the leaf photosynthetic capacity can be genetically improved in crop species based on the recent progress regarding physiology and genetics. Some traits that are outside of the scope of this paper, such as kinetics and activity of Rubisco, are also important for improving the leaf photosynthetic rate (Suzuki et al., 2009; Ishikawa et al., 2011; Whitney et al., 2011). With respect to methodology, techniques allowing the rapid visualization of photosynthesis, such as thermography (Maes and Steppe, 2012), imaging-PAM (Baker and Rosenqvist, 2004; Omasa et al., 2009) or the direct quantification of O$_2$ (Tschiersch et al., 2012), are also expected to accelerate photosynthesis research. However, the fact that real photosynthesis is highly heterogeneous over space and time should not be overlooked. For instance, the effects of the leaf position and nutrient availability make this matter complex. This complexity may be one reason for the ‘missing link’ between leaf photosynthesis and crop productivity. Moreover, the photosynthetic adaptation of crop plants to future climate changes, such as high CO$_2$ concentrations, rising temperatures, more frequent extreme temperature events, and precipitation changes, will represent a significant challenge for photosynthesis physiologists. Hence, the issue should not be ‘how to configure a leaf with improved photosynthetic performance’, but ‘how the configured leaf performs under various environments’. From this view point, the physiological study of leaf photosynthesis will be revalued in crop science.

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