Induced Genetic Variations in Stomatal Density and Size of Rice Strongly Affect Water-Use Efficiency, Drought Tolerance, and Responses to Abiotic Stresses

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Abstract

Background

Rice (*Oryza sativa*) is one of the world's most important crops and is especially important in Asia. Because irrigated rice consumes about 34–43% of the total water used for irrigation globally, increasing drought and global temperature will increase the risk of rice crop loss. However, rice is among the least efficient crops in terms of water use, whereby most of the uptaken water is used for transpirational cooling via stomatal pores on the leaf blades and sheaths. To increase the water-use efficiency of rice, alterations in stomatal density and size may help to reduce transpiration and thereby increase water-use efficiency (WUE).

Results

We identified four stomatal model lines—with either high-density (HD) or low-density (LD) stomata and small-sized (SS) or big-sized (BS) stomata—from the Mutant Core Collection (MCC) of 216 mutants. Gas exchange analysis revealed that the stomatal model lines have similar photosynthetic assimilation (A) and chlorophyll fluorescence. With increasing CO_2 concentration, A of all stomatal model lines was observed to respond similarly at 100–600 ppm CO_2 , but beyond this point, SS was more responsive to increasing CO_2 concentration than the other stomatal model lines. HD had higher stomatal conductance (g_s) and g_{smax} than the other stomatal model lines. In response to a mild heat at 30 °C, both SS and LD had higher canopy temperature than HD, BS, and JHN-wt as a result of heat retention. All stomatal model lines are also similar in their rhythmic stomatal responses to ten-minute dark/light transition cycles, except that initial stomata closure in SS was more rapid than in BS.

The stomatal model lines did not show any significant differences in the response to short-term water stress. Long-term water stress had less impact on leaf drying, F_v/F_m , grain yield, and harvest index in LD and SS. In the field, all stomatal model lines and JHN-wt had similar WUE in the sufficient-water treatment. LD had the highest WUE and biomass/plant than any stomatal model lines in the long-term restricted-water treatment.

Conclusions

Taken together, our results suggest that induced alterations in stomata density and size influence rice WUE and the responses to drought and heat stresses, providing further understanding of the roles of stomata density and size in related processes. The low-density and small stomata lines have high potential as genetic donors for improving WUE and drought in climate-ready rice.

Background

Rice (*Oryza sativa* L.) is a major crop, providing food for billions of people globally (Elert, 2014; Redfern et al., 2012a). However, its cultivation is water intensive, requiring approximately 2500 liters of water per

kilogram of polished grain produced (Bouman, 2009). Most of this water is utilized by rice for transpiration through stomata pores, and considering the low water-reserving efficiency, rice is extremely vulnerable to climate change (Meyer, 2014 Caine, 2018) (Meyer et al., 2014; UNESCO, 2016; Yao et al., 2017; Caine et al., 2018). Drought and heat are two major threats to rice growth and development (Solomon et al., 2009; Jagadish et al., 2015; Korres et al., 2017; Kumar et al., 2017; Caine et al., 2018). Rice has amongst the highest stomata density and smallest stomata size of all plant species (Bertolino et al., 2019). Rice leaf regulates gaseous exchange and transpiration with the environment via stomatal complexes, which predominantly develop in files on the epidermis (Hepworth et al., 2018; Luo et al., 2012). Each stomatal complex consists of a pair of dumbbell-shaped guard cells that are surrounded by specialized epidermal cells called subsidiary cells, which may aid in increasing the efficiency of opening and closing (Chen et al., 2017; Raissig et al., 2017). Because the important functions of stomata are gas exchange and transpiration, altering stomatal traits such as density and size is hypothesized to be the key to improving WUE (Bertolino et al., 2019).

Plants capture CO₂ for photosynthesis by increasing stomatal aperture with the concurrent release of water, enabling plant cooling when temperatures are high (Caine et al., 2018; Mustilli et al., 2002; Zeiger et al., 1987). During short-term drought, reductions in stomatal aperture restrict CO₂ uptake and water release, leading to increased plant temperature (Hepworth et al., 2015; Raven, 2014). A study on rice that was 49–62 days old with low stomata density found that it was cooler than control rice with high stomata density under the same water-stress conditions (Caine et al., 2018).

Throughout the entire cycle of plant growth, stomatal development is adjusted to fit the surrounding environment, often by modifying the density and size of stomata on the plant epidermis (Caine et al., 2018; Casson and Gray, 2008; Mustilli et al., 2002). For example, exposure to prolonged high light, high humidity, or low atmospheric CO₂ concentrations often leads to increased stomatal density (Casson, 2009, Tricker, 2012), whereas drought, low light, or increased atmospheric CO₂ concentration reduce stomatal density (Casson and Gray, 2008; Elert, 2014; Hamanishi et al., 2012; Hetherington and Woodward, 2003). When the temperature is increased, the stomatal density is reduced in *Arabidopsis* (Crawford et al., 2012) but increased in rice (Caine et al., 2018).

Recent research examining stomatal responsiveness to light across a range of plant species showed that the small dumbbell-shaped stomata of grass species respond faster than large dumbbell-shaped stomata; for plants with elliptical-shaped stomata, size does not correlate with stomatal response speed (Lawson and Vialet-Chabrand, 2018; Mcausland et al., 2016). A recent study of SLAC1-deficient rice mutant revealed it exhibited significantly faster stomatal opening upon an increase in light irradiance, which led to faster photosynthetic induction (Yamori et al., 2020). By more rapidly altering stomatal aperture in response to light, the g_s of small dumbbell-shaped stomata was more rapidly altered, leading to improved iWUE. When the stomatal densities of grass species with small dumbbell-shaped stomata were assessed, no negative correlation was found between stomatal size and density, except for rice. Translational research related to the regulation of stomatal development in grasses is rapidly expanding,

with a number of researchers analyzing the physiological effects of altering stomata (Caine et al., 2018; Hughes et al., 2017; Raissig et al., 2017; Schuler et al., 2018). In *Arabidopsis* and rice, stomatal density is controlled by a single gene that regulates the development of epidermal cells (Sugimoto et al., 2013; Bertolino et al., 2019). Overexpressing EPF1 markedly reduced stomata density and improved iWUE in barley and rice (Caine et al., 2018; Hughes et al., 2017). Therefore, variation in stomatal density and size can alter *A* and *g*, leading to changes in iWUE.

The success of conventional breeding and marker-assisted selection relies heavily on available genetic variation. The depletion of genetic diversity is therefore a crucial limiting factor for marker-assisted breeding in helping to feed the increasing world population. Mutagenesis induced by radiation or chemicals can rapidly trigger structural and nucleotide changes in the genome, which consequently induce both random and spontaneous mutations that may lead to phenotypic gain and be used in breeding programs (Reungphayak et al., 2015; Kamolsukurnyong et al., 2019). In this study, we used the Mutant Core Collection (MCC), which is a JHN purple rice cultivar population whereby large-scale, long-term mutations were induced using fast-neutron radiation (Ruengphayak et al., 2015). This collection was established for the purpose of discovering unique genetic variations that could be used to establish nutritionally dense rice that can counter biotic and or abiotic stresses. Here, we screened the Mutant Core Collection (MCC), consisting of 216 M₆ mutants selected based on extreme phenotypic variation, to find rice plants with altered stomatal size or density. We selected four model lines representing extreme stomatal traits, including high stomata density (HD), low stomata density (LD), big stomata size (BS), and small stomata size (SS). We also examined how modifying stomatal size and/or density impacts gas exchange, plant temperature, and response to drought in an attempt to generate traits that will be beneficial to farmers over the coming years and decades.

Results

Mutants with Altered Stomatal Density/Size

Stomata can be detected on both the adaxial and abaxial surfaces of rice leaf and form in rows alongside vertical leaf veins (**Fig. 1**). To identify mutants with altered stomatal phenotypes, we screened the flag leaves of all 216 members of the MCC by microscopic observation. The Mutant Core Collection comprises all selected mutants resulting from previous forward and reverse screening of the large fast-neutron mutagenized population. Because fast-neutron mutagenesis has the tendency to induce mutation hotspots of multiple traits on specific lines, the MCC represents highly mutagenic mutants with a high chance of finding more mutable traits. The results revealed that 7.8% and 3.7% of the MCC lines showed variation in stomata density and size, respectively. From the 216 MCC members, 6 and 11 mutant lines either showed increased and reduced stomata density, respectively (Table 1). Five and three mutants were found in terms of having respectively increased or decreased stomata size. Stomata density tended to be higher on the abaxial than on the adaxial surface. However, a larger alteration in stomata density was detected on the adaxial side ($401\text{--}869\text{ mm}^{-2}$) than on the abaxial side ($284\text{--}597\text{ mm}^{-2}$) (**Fig. 1c,d**). As such, no correlation was detected between leaf side and stomata densities. The results also showed

that stomatal density and size are not correlated (**Supplementary Table 1, Figure 3**), and the only correlation found was between stomata size ($R^2 = 0.48$) (**Fig. 1a,b**).

We selected four stomatal model lines that clearly showed altered stomatal traits, small size (SS), big size (BS), low density (LD), and high density (HD), as a platform for gene identification and to understand the physiological impact of these alterations on photosynthesis, water-use efficiency, and tolerance to drought and heat stresses (**Fig. 1c,d,e-i**) (**Fig. 1d** and **Supplementary info. 1, Figure 2a,b**). To confirm the stability of the altered stomatal phenotypes in the four stomatal models, we evaluated the stomatal traits on flag leaves grown under sufficient-water and restricted-water conditions. The differences in stomata density and size were more pronounced in sufficient-water than in restricted-water conditions (data not shown).

Stomatal conductance and canopy temperature

To understand how changes in stomatal size and density affect A and g_s , we measured gas exchange using an infrared gas analyzer set at saturating light conditions ($2000 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR) among the stomatal model lines that were distinctive for stomatal density or size. We found no significant differences in A or chlorophyll fluorescence among the stomatal model lines and JHN-wt (**Fig. 2e** and **Supplementary Fig. 4**). For stomatal conductance, HD has significantly higher g_s and $g_{s\text{max}}$ than BS. By contrast, both LD and SS are similar in gas exchange, having low g_s and the lowest $g_{s\text{max}}$, despite the clear differences in stomata density or size. Interestingly, despite the low g_s , BS had the second highest $g_{s\text{max}}$, highlighting a wide disparity between actual g_s and $g_{s\text{max}}$ (**Fig. 2f,h**). Both SS and LD had a higher canopy temperature than HD, BS, and JHN-wt in response to mild heat stress at 30°C (**Fig. 2d**). This may reflect a lower rate of transpiration in SS and LD than in HD and BS. Therefore, the variation in stomata size and density, such demonstrated by SS and LD, may reflect restrictive transpirational cooling but not necessarily stomatal conductance and photosynthesis, even under mild heat stress (**Fig 2c,d**) (ANOVA, $P \leq 0.05$).

Response to increased atmospheric CO_2

One of the main causes of global warming is the increasing amounts of greenhouse gases (GHGs), such as CO_2 , released to the atmosphere. To understand if the alteration in the stomata density or size affects the response to increasing CO_2 concentration, the assimilation rates of the stomatal model lines were evaluated. As the CO_2 increased from 100 to 1400 ppm, A gradually increased to a maximum at 600 ppm and then slowly declined as the CO_2 approached 1500 ppm. The stomatal model lines were not significantly different at 100–600 ppm but became more distinctive beyond 600 ppm (**Fig. 3**). At these high CO_2 concentrations, SS and HD showed higher A than LD and BS. SS performed the best, followed by HD, in the response to the above-ambient CO_2 concentrations (**Fig. 3**). Under such conditions, the difference in A between SS and BS was clearly detected, showing that mild heat stress affects SS and LD.

Therefore, optimized rice varieties with small and highly dense stomata may be more beneficial for increasing biomass and grain yield under future climate change.

Rhythmic stomatal response

We evaluated the impact of altered stomata traits on the rate of stomatal closure and re-opening in response to a rapid dark/light transition (**Fig. 4**). The stomatal model lines were evaluated for rhythmic stomatal functioning by monitoring the changes in g_s in 10 min dark/light intervals. For each cycle, when the light was turned off, g_s slowly declined until 10 min; when the light was turned on, g_s quickly continually increased up to the end of the 10 min period or illumination. When comparing LD, HD, and JHN wt, no significant differences in g_s were detected across any timelines, suggesting that variation in stomata density does not have any effect on the rhythmic stomata response (**Fig. 4g-i**). However, SS was significantly faster than BS during the initial 5-minute dark period only (ANOVA, $P < 0.05$) (**Fig. 4c**). Beyond that point, no significant differences were detected, suggesting that stomata size does not have strong effect on rhythmic response to dark/light transitions (**Fig. 4c-f**).

Drought response

Having measured various physiological parameters related to stomatal functioning, we next wanted to understand whether alterations in stomatal density and size had any impact on drought tolerance and WUE. Under sufficient-water conditions, all stomatal model lines were similar in biomass and grain yield (**Fig. 5a,c**). HD had a significantly higher yield than BS ($P < 0.05$) but not LD and SS under mild water stress conditions (5D-W-5D) (**Fig. 5d**). Under long-term drought conditions, 5D-14W, water was withheld for 5 days at panicle initiation, followed by the addition of 200 mL water every other day for 14 days. This treatment created more severe water stress than the 5D-W-5D treatment, which caused leaf drying (%DL) in all stomatal model lines and JHN-wt. LD and SS showed less %DL than BS, HD, and JHN-wt on 7th and 14th days after withholding water. LD and SS produced higher quantum yield (F_v/F_m), grain yield, and HI under the long-term water stress treatment. When compared to the sufficient-water treatment, LD and SS showed less reduction in F_v/F_m , grain yield, and HI than BS, HD, and JHN-wt under long-term severe water stress (**Fig. 6c-e**). These results suggest that LD and SS are less sensitive to long-term water stress than HD and BS.

To determine if the alteration in stomatal density and size affected WUE, the stomatal model lines were evaluated on outdoor large cement blocks to effectively control irrigation throughout the whole plant development cycle. Restricted water severely affected percent seed set, panicle/plant, biomass/plant, and WUE (**Table 2**). Under the sufficient-water treatment, all stomatal model lines showed similar WUE as JHN-wt. By contrast, the restricted-water treatment severely affected HD in terms of percent seed set, panicle/plant, biomass/plant, and WUE. LD performed better than HD for every agronomic trait and showed the highest biomass and WUE of all studied stomatal model lines. As LD maintained quantum yield, harvest index, biomass, grain yield, and WUE in such long-term water stress conditions, the results

suggest that rice breeders should pay more attention to reducing stomata density when developing new water-use-efficient rice to save irrigation water and improve drought tolerance in rice.

Discussion

Mutation induced for stomata alteration

In the current study, we demonstrated that fast-neutron-induced mutagenesis allows breeders to successfully apply useful genetic variation that is not possible with the narrow-genetic-based breeding currently used in rice. Here, the stomatal model lines that express distinctive stomata density or size on an otherwise similar genetic background provided the platform to understanding the impact of stomatal traits on abiotic stress responses. Despite the relatively large differences in density and size detected on flag leaves, the genetic control of stomata density and size is developmentally and environmentally regulated during vegetative growth. Unlike EPF1-overexpressed IR64, reduced stomatal density exhibited consistent differences in stomata traits throughout plant development (Caines et al., 2018). This suggests that the genetic regulators of altered stomata traits induced by mutation and genetic engineering did not originate from the same genetic factors (Doheny-Adams et al., 2012; Franks and Beerling, 2009).

Stomatal variation influences rice temperature

Rice cultivation has been increasingly affected by global warming and climate change (Jagadish et al., 2015; Meyer et al., 2014; Redfern et al., 2012b). The global rise in temperature has reached about 0.2 °C per decade and may reach 1.5 °C above the pre-industrial temperature by 2040 (The State of the Global Climate 2020, World Meteorological Organization, 2021). Because rice consumes approximately 2.5 tons of water to produce only 1 kg of grain, the water-use efficiency of rice is low (Bouman 2009). Of the water that is consumed, rice transpires 500–1000 kg or 20–40% of the total consumed to the atmosphere (Bouman 2009). We hypothesize that rice is actually heat sensitive throughout its life cycle and, therefore, uses transpirational cooling as the key mechanism to overcome heat stress. Stomata, small pore-forming cell structures on the rice leaf and leaf sheath, play vital roles in controlling transpirational cooling and gas exchange. Among crop plants, rice has not only the highest stomatal density but also the smallest stomata (Mcausland et al., 2016). To understand the physiological responses to heat and water stresses, lines with genetic variations in stomatal traits, such as density and size, have been developed. Studies on these lines showed that altered stomata density and size affect physiological responses to heat and water stresses. In IR64, the reduction in stomata density caused by overexpression of OsEPF1 resulted in plants that were 0.3 °C warmer in sufficient-water treatment but cooler than the respective IR64 controls during the vegetative stage in a restricted-water treatment (Caine et al., 2018). By contrast, our results using the stomatal model lines showed that small stomata size (SS) resulted in warmer rice compared to big stomata size (BS) and high stomata density (HD) even under mild heat (30 °C). We found that only low stomata density (LD) had less impact than smaller stomata size (SS) on canopy temperature; stomatal density had no impact on canopy temperature under mild heat and sufficient water conditions.

The stomata density of transgenic IR64OsEPF1 was 51–74% lower than that of IR64-wt (Caine et al., 2018). More studies are needed to understand the effects of stomata size on canopy temperature at high ambient temperatures.

Smaller stomata respond more rapidly

Recent work across a range of plant species revealed that the small dumbbell-shaped stomata of grass species respond quickly to changing light intensity (Lawson and Vialet-Chabrand, 2018; Mcausland et al., 2016). The speed at which stomata can respond to the changing environment is important to improving short-term WUE (Lawson and Vialet-Chabrand, 2018; Mcausland et al., 2016). It has been shown that plants with smaller stomata size and, in some cases, high-density stomata have a higher dynamic range of g_s , which permits leaves to adapt more quickly to changing environments, such as temporary shading (Drake et al., 2013; Lawson and Vialet-Chabrand, 2018; Mcausland et al., 2016; Ohsumi et al., 2007). In our stomatal model lines, density did not affect the response speed of stomatal aperture. Instead, small stomata were significantly faster than larger stomata only for initially closing. It would be interesting to test whether smaller stomata can still produce a similar response under low light intensity or shading.

Lower stomata density leads to increased water-use efficiency and survival under long-term drought

In the short-term drought experiment, we did not detect any significant differences in yield or biomass. However, under long-term drought conditions, rice with smaller stomata and lower density showed comparatively less damage and less reduction in quantum yield (F_v/F_m). As such, rice lines with smaller and lower density stomata are more adaptive to drought stresses and able to maintain biomass, grain yield, and harvest index values. The results from the IR64-OsEPFoe lines support the finding that lower stomata density reduces water use by 60% under drought conditions during the seedling stage (Caine et al., 2018). To link WUE to alteration in stomata traits, we tested the stomatal model lines in irrigation-controlled cement blocks where 908 and 348 L of irrigation water were applied in sufficient-water and restricted-water treatments, respectively. Such irrigation treatments significantly affected percent seed set, panicles/plant, biomass/plant, and WUE. However, no differences were observed in the WUE among the stomatal model lines in the sufficient-water condition. Only in the restricted-water conditions did plants with lower stomata density (LD) show better growth than those of the other stomatal model lines. As such, rice with lower stomata density (LD) produced 34% more biomass than higher stomata density (HD) with the same amount of irrigation water applied under restricted-water conditions. In addition, the results of this study show that having lower stomata density (LD) resulted in more stable growth under long-term drought conditions as measured by photosynthetic assimilation, stomatal conductance, chlorophyll fluorescence, harvest index, and grain yield.

Conclusions

In this study, we demonstrated the genetic potential of fast-neutron mutagenesis to rapidly induce genetic variation. The stomatal density and size were independently induced at high frequency. We used four

stomatal mutant lines, each showing distinct stomatal phenotypes, to evaluate the impact of altered stomata density and size on the responses to abiotic stresses and water-use efficiency. The stomatal model lines share a similar photosynthetic assimilation rate (A). The line with high stomata density (HD) had the highest stomatal conductance. Small stomata respond faster to dark/light cycles and better to high CO₂ concentration gradients above 600 ppm but retain more heat under mild heat stress and sufficient-water conditions. In the long-term drought conditions, small stomata and low stomata density were associated with lower leaf drying and less reduction in grain yield, harvest index, and F_v/F_m . In the field, low stomata density had a strong impact on improving biomass production and water-use efficiency. Therefore, reducing stomata density will empower plant breeders to successfully develop climate-ready rice to lessen the impact of drought and heat stresses and the imminent threat of global warming.

Materials And Methods

Mutant Population

The fast-neutron library was developed from the photoperiod-insensitive, purple rice variety Jao Hom Nin (JHN), by Rice Science Center, Kasetsart University, Thailand, as previously described (Ruengphayak et al., 2015).

Stomatal variation screening

The rice plants used in this study were 216 mutant lines of the JHN Mutant Core Collection. All rice lines were screened by observing the stomatal characteristic under a compound light microscope. Candidate lines identified in the study were used to evaluate drought tolerance.

Phenotypic variation in stomatal traits

To identify plants with stomatal phenotypes from the mutant population, we screened the flag leaves of 216 Mutant Core Collection members by measuring the stomatal size (guard cell length) and density on 3 biological replicates per line (Fig. 1, Supplementary Fig. 1). Stomatal traits of the 216 mutants derived from an indica rice cv. Jao Hom Nin were screened, yielding four candidate lines that have specific stomata traits. Fully expanded leaves were collected from the 216 Mutant Core Collection members grown in the experimental field at the Kasetsart University, Kamphaeng Saen campus. Stomata were localized in rows parallel to leaf veins (Figure 3).

Microscopic observation of stomatal traits

Leaf sections from the adaxial and abaxial leaf surfaces were imprinted on glass slides by applying dental resin (Coltene Whaledent, Switzerland) or nail varnish peels over a fully expanded leaf surface to achieve leaf imprinting. Cell counts were captured from the middle area of leaves from at least eight plants per genotype and using six fields of view per leaf. Leaf epidermal imaging was conducted using a

camera fitted on a light microscope (Leica, DM750-ICC50 HD) and ImageJ software (Fiji v. 1.51u). Stomatal density within each field of view was counted manually and total stomatal density per mm² of leaf was calculated by applying a set conversion factor. Stomatal size was determined by measuring the length of the guard cell from tip to base; a minimum of 10 randomly selected stomata were measured. Total stomatal complex area was calculated by manually tracing around the outside of both the guard cells and subsidiary cells using the polygon tool in ImageJ. Further stomatal measurements, such as pore length and guard cell width, were also measured and used to calculate the maximum pore aperture and the anatomical maximum stomatal conductance using the Franks and Farquhar (2001) equation.

Plant material and growth conditions

For the plant growth of the four selected FNB lines and the JHN control line, rice seeds from the M₅ generation were placed into sealed Petri dishes containing sterile water and kept at room temperature (25–30 °C) with ample light. One-week-old seedlings were transplanted into pots containing soil originating from the same field as the initial phenotyping. We carefully added the same amount of soil to each pot. Plants were cultivated in the greenhouse with temperatures averaging 30–40 °C. Eight plants of each line were grown, and phenotypic measurements were performed on flag leaves between November and December 2017. Organic fertilizer was applied twice during plant development, at the late seedling stage and the maximum tillering stage. This plant set was prepared for the short-term drought experiment and leaf gas exchange measurement.

Leaf gas exchange measurement at steady state and g_{smax} calculation

Stomata conductance and light response assays were performed using a LI-6400 portable photosynthesis system (Licor, Lincoln), conducting infrared gas analysis (IRGA) on the first fully expanded flag leaf from the primary tiller using an LI-6400XT portable photosynthesis system. For heat experiments, an LI-6800 (Licor, Lincoln) was used with a chamber flow rate of 400 $\mu\text{mol s}^{-1}$ and leaf temperature of 32 °C. Reference CO₂ was maintained at 480 ppm and light intensity at 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The relative humidity inside the IRGA chamber was kept at 65–75% using a self-indicating desiccant. Steady-state measurements under these conditions were recorded for carbon assimilation (A) and stomatal conductance (g_s) for the restricted-water experiment; A and g_s were measured between 9 a.m. and 12 p.m. on 7th and 14th days after water stress treatments. Intrinsic water-use efficiency (iWUE) was calculated from carbon assimilation divided by stomata conductance (A/g_s). Abaxial anatomical g_{smax} was calculated using the double end corrected version of the Franks and Farquhar (2001) equation, from Dow et al. (2014):

$$\text{Anatomical } g_{smax}: (d \cdot D \cdot a_{max}) / (v \cdot (l + \pi/2) \cdot \sqrt{(d_{max}/\pi)}),$$

where ($\text{m}^2 \text{s}^{-1}$) is the diffusivity of water in air and v ($\text{m}^3 \text{mol}^{-1}$) is the molar volume of air. Assuming equal stomatal densities on both sides of the leaf, this value was doubled to provide the total anatomical

Rhythmic response to dark/light transition

The experiments were conducted in the greenhouse, where plants were acclimatized at saturating light ($2000 \mu\text{mol m}^{-2} \text{s}^{-1}$), followed by 10 minutes of complete darkness ($0 \mu\text{mol m}^{-2} \text{s}^{-1}$), and then saturating light was re-applied for the final 10 minutes. At each light intensity, 20 measurements for A and g_s were recorded. Stomatal closure was instigated by 10 minutes of complete darkness ($0 \text{ m}^{-2} \text{s}^{-1}$ PAR) following steady-state conditions of saturating light ($2000 \text{ m}^{-2} \text{s}^{-1}$ PAR). To re-open stomata, saturating light was re-introduced. The rate of g_s per minute of the stomatal model lines were analyzed in 5-minute segments during initial stomatal closure, late stomatal closure, initial stomatal opening, and late stomatal opening.

CO₂ Assimilation rate (A) response to different ambient CO₂ concentrations

A was measured for the following carbon dioxide values: 100, 200, 340, 480, 600, 800, 1000, 1200, and 1500 ppm. At each step, a pause of 10 min was allowed for A to stabilize before the measurements were automatically recorded.

Water stress experiments

Experiment I: Panicle-initiation stage (R1) 5D-W-5D

The first experiment was conducted during the reproductive stage, when flag leaves were fully expanded and the panicle was initiated. Initially, plants were kept sufficiently watered for 82 days after transplantation. Then, a subset of plants were restricted-water treated by withholding water for 5 days, followed by re-watering to saturate the soil, and then withholding water for another 5–10 days. The normal control groups were kept well-watered throughout the experiment. Once the plant had reached maturity, all the plants were harvested to determine the aboveground wet biomass, grain yield, and total weight of filled seeds for each plant.

Experiment II: R2 reproductive water stress (R1-2 5D-14W)

The water was drained before the R2 stage for 3 days or at 71 days after transplantation, withholding water for 5 days, and then adding 200 mL of water every 2 days until 14 days. All physiological parameters and plant phenotypes were measured 7 and 14 days after treatment. Leaf dryness percentage (%DL) scorings were divided into four levels as: highly tolerant (0–25%), tolerant (26–50%), moderate (51–75%), and sensitive (76–100%) to drought.

High-resolution phenotyping

HRPP was performed at nighttime for chlorophyll fluorescence, thermal imaging, and hyperspectral imaging.

1. **Estimation of quantum yield of photosystem II (Φ PSII):** The quantum yield was measured using a PSI FluorCam FC-800MF pulse amplitude modulator installed on the system. A FluorCam was used to capture chlorophyll fluorescence images and to estimate the maximum quantum yield of PSII (F_v/F_m) of the control and treated leaves. Fluorescence chlorophyll imaging was set up without dark adaptation daily in the early morning (6 a.m.) during the five days of drought measurement. Raw data were analyzed and images were acquired using the Plant Data Analyzer program. Plant vegetative biomass was also measured using the wet tissue of the plants, and the tiller and panicle numbers of the plant were recorded as well as the yield harvested. PSI Open FluorCam FC 800-O (PSI, Brno, Czech Republic) was used to capture the chlorophyll fluorescence images and to estimate the maximum quantum yield of PSII (F_v/F_m) in the leaves of control and treated plants. For chlorophyll fluorescence, the minimum fluorescence (Crawford et al.) and maximum fluorescence (F_m) in a dark-adapted state were used to calculate the maximum PSII quantum yield (QY) using the equations:

$$QY = F_m - F_o/F_m$$

$$QY = F_v/F_m$$

2. For all image acquisitions, observed leaves were maintained horizontally and the images were acquired and analyzed using the Fluorcam 7 program (PSI).
3. **Canopy temperature:** One day before the drought experiments, canopy temperature was evaluated using specially designed FLIR thermal cameras on the Plants Screen System (PSI, Brno, Czech Republic) with a resolution of 640 × 480 pixels.

Water-Use Efficiency: Rice seeds were germinated on 200-well plastic plates. At 21 days, seedlings were transferred into 1.2 m (diameter) × 0.8 m (height) round concrete blocks (CBs), which held 750 kg of paddy soil mix per block. We grew 24 seedlings with 20 cm spacing on each CB until harvest (Figure 2.2.6.). Soil temperature was measured 12 cm below the soil surface. A split-plot design with the main-plot, sufficient-water, and restricted-water treatments were set with four replicates using a randomized complete block (RCB) design. Phenotypic data were collected weekly. Soil moisture and temperature were monitored daily at 3:00 p.m. at a 12 cm soil depth in each CB using a soil moisture meter (Field Scout TDR150). From germination to maximum tillering, irrigation for all treatments provided sufficient water until the initiation of the reproductive stage. Five liters of irrigation were applied daily to each CB until the initiation of the reproductive stage. The average total amount of water use during the vegetative stage under both conditions was 340 L/CB. Then, the irrigation was increased to 10 L/CB daily during the reproductive period when rice uses more water for grain filling in the sufficient-water treatment. No irrigation was applied to the restricted-water plants. However, 8 L of rainfall was added to each CB during

the experiment. The average volumetric soil moisture content (VMC) of sufficient-water and restricted-water treatments were 35–45% and 10%, respectively. The average soil temperatures of sufficient-water and restricted-water treatments were 35–37 and 40–43 °C, respectively, throughout the growing season. When rice is approaching maturity, the number of panicles per cm² and 1000 seed weight were collected for each line. Seed set percentage is defined as:

$$\% \text{ seed set} = (\text{seed-filled florets} / \text{total florets}) \times 100$$

Biomass is the dried weight of the whole aboveground plant.

Water use efficiency (WUE) is the dried weight of biomass (kg) per liter of water used. The total amount of water used in the sufficient-water and restricted-water treatments was 908 and 348 L/CB, respectively.

Statistical analysis

One-way ANOVA was used to determine if there were interactions between treatments. Treatment means were compared using least significant difference (LSD) to determine whether they were significantly different at the 0.05 probability level.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its supplementary files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AV, JG, SA conceived the concept of the experiment and this manuscript; MP, RC, CH, EH, JS, CC, CP, and RN performed the experiments; MP and AV wrote the manuscript; SR maintained the JHN mutant population; MP and AV analyzed the data; AV revised the manuscript and all the authors reviewed and approved this submission.

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Tables

Table 1. Outcomes of the microscopic screening of stomatal density/size in the 216-member Mutant Core Collection.

Trait	Pop (n)	Selected mutants		Approach
		Increase	Decrease	
Stomata density	Core (216)	6	11	No. of stomata per 12 m ²
Stomata size	Core (216)	5	3	Length of guard cell (cm)

Table 2. The % seed set, panicle/plant, biomass/plant, and water-used efficiency (WUE) among stomatal model lines and JHN wt grown in sufficient-water and restricted-water conditions.

Treatment	Lines	% seed set		Panicle/plant		Biomass/plant		WUE g/L	
Sufficient water	JHN	84.50	a	16.23	ab	40.81	ab	0.61	e
Sufficient water	BS	84.20	a	17.43	a	39.81	abc	0.60	e
Sufficient water	HD	71.48	c	12.00	cd	40.31	abc	0.61	e
Sufficient water	SS	86.70	a	15.65	b	37.91	c	0.57	e
Sufficient-water	LD	79.18	b	15.58	b	38.36	bc	0.58	e
Restricted water	JHN	35.60	f	5.60	g	20.51	fg	0.80	b
Restricted water	BS	35.55	f	5.75	fg	18.55	gh	0.73	bc
Restricted water	HD	28.15	g	6.68	ef	17.50	h	0.69	cd
Restricted water	SS	39.53	def	7.65	e	20.13	g	0.79	b
Restricted water	LD	36.48	ef	7.50	e	23.69	de	0.93	a
Mean									
Sufficient water		81.12	a	15.38	a	39.38	a	0.59	b
Restricted water		35.60	B	6.64	B	20.08	B	0.79	a
F-test									
Water conditions		**		**		**		**	
Line		**		**		**		**	
Water condition × Line		**		**		**		**	

Figures

Figure 1

Stomatal size and density screening using the flag leaves of 216 MCC members. XY scatter plots of abaxial and adaxial (a) stomatal size and (d) stomatal density. Distribution of the 216 MCC lines according to (c) abaxial stomatal size and (d) abaxial stomatal density, where significant correlations were observed ($P < 0.001$). (e-i) Representative illustrative drawings based on tracings of the (e) JHN wt and (f-i) lines identified as have (f) small (SS) or (g) big stomata (BS), or (h) low (LD) or (i) high stomatal

density (HD). Guard cells are indicated in yellow, and subsidiary cells, in red. The bin location of the four stomatal model mutant lines along with JHN-wt are marked in c and d (scale bars = 25 μm).

Figure 2

Whole-plant temperature and gas exchange analysis of stomatal model lines with altered density or size. Flag leaf (a) stomatal size and (b) density measurements conducted on all stomatal model lines. (c) Representative thermal image of JHN-wt and all stomatal model lines (d). Quantification of plant

temperature based on the thermal images in (c). (e) Carbon assimilation (A) and (f) stomatal conductance of stomatal model lines under saturating light conditions (PAR 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$). (g) g_s max of JHN-wt and stomatal model lines. (a,b,d-g) Boxes indicate the upper (75%) and lower (25%) quartiles, and horizontal lines within the boxes indicate the median. Whiskers indicate the ranges of the minimum and maximum values, and different letters within a graph indicate a significant difference between the means of $P < 0.05$ (ANOVA, post hoc multiple comparisons, Holm–Sidak method). (a-d,g) $n = 8$; (e,f) $n = 4-5$.

Figure 3

Assimilation (A) in response to changing carbon supplies (C_i) of four mutant lines: JHN826 (BS) JHN3117 (SS), JHN2447 (HD) and JHN8756 (LD).

Figure 4

Stomatal conductance responses to dark/light cycle over 20 minutes in terms of the (a) equivalent g_s response for SS, BS, and JHN-wt (c-f); and the (b) equivalent g_s response for LD, HD, and JHN-wt (g-j). (a,b) Error bars = s.e.m. (c-j) Boxes indicate the upper (75%) and lower (25%) quartiles, and horizontal lines within a box represent the median.

Figure 5

The above ground biomass of stomatal model lines and JHN-wt subject to 5D-W-5D short-term water stress treatment were compared under (a) sufficient-water and (b) drought conditions. Grain yield under (c) sufficient-water and (d) drought conditions. In all graphs, boxes indicate the upper (75%) and lower (25%) quartiles, and horizontal lines within boxes indicate the medians. Whiskers indicate the ranges of the minimum and maximum values, and different letters within a graph indicate a significant difference between the means of at least $P < 0.05$ (ANOVA, post hoc multiple comparisons, Holm–Sidak method; $n = 6$ plants).

Figure 6

In 5D-14W, %DL and yield performance of stomatal model lines and JHN-wt under conditions of sufficient water and 14 days withholding water: (a) %DL observed 7 days after drought, (b) leaf drying observed 14 days after drought, (c) total yield measured for both sufficient-water (gray) and drought conditions (pink), (d) harvest index for both sufficient-water (gray) and drought conditions (pink), and (e) Fv/Fm measured 14 days after drought comparing sufficient-water (gray) and drought (pink) conditions. Whiskers indicate the ranges of the minimum and maximum values, and different letters within a graph indicate a significant difference between the means. The numbers on top of the bar in the lower graphs indicate the reduction percentage. Statistical analysis was conducted using R statistical software.

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