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Rice leaf hydrophobicity and gas films are conferred by a wax synthesis gene (LGF1) and contribute to flood tolerance

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Summary

- Floods impede gas (O2 and CO2) exchange between plants and the environment. A mechanism to enhance plant gas exchange under water comprises gas films on hydrophobic leaves, but the genetic regulation of this mechanism is unknown.
- We used a rice mutant (dripping wet leaf 7, drp7) which does not retain gas films on leaves, and its wild-type (Kinmaze), in gene discovery for this trait. Gene complementation was tested in transgenic lines. Functional properties of leaves as related to gas film retention and underwater photosynthesis were evaluated.
- Leaf Gas Film 1 (LGF1) was identified as the gene determining leaf gas films. LGF1 regulates C30 primary alcohol synthesis, which is necessary for abundant epicuticular wax platelets, leaf hydrophobicity and gas films on submerged leaves. This trait enhanced underwater photosynthesis 8.2-fold and contributes to submergence tolerance. Gene function was verified by a complementation test of LGF1 expressed in the drp7 mutant background, which restored C30 primary alcohol synthesis, wax platelet abundance, leaf hydrophobicity, gas film retention, and underwater photosynthesis.
- The discovery of LGF1 provides an opportunity to better understand variation amongst rice genotypes for gas film retention ability and to target various alleles in breeding for improved submergence tolerance for yield stability in flood-prone areas.

Introduction

Water limits global agricultural production; too little or too much destroys crops. Floods alone can cause yield losses worth $US 7.8 billion every year (FAO, 2015). Even rice (Oryza sativa), a wetland crop which grows in shallow standing water and feeds a large proportion of the world population, frequently suffers damage or loss in flood-prone regions (Ismail et al., 2013). Inundation by water impedes shoot gas exchange, which slows CO2 entry and restricts photosynthesis during the day and slows O2 uptake, resulting in tissue hypoxia during the night (Pedersen et al., 2009; Colmer et al., 2014). A mechanism to enhance tissue gas exchange with water, evident for both aquatic invertebrate animals and plants, comprises hydrophobic body surfaces that
retain a thin layer of gas; for plants, the term ‘leaf gas films’ has been used to describe this trait (Pedersen et al., 2009; Pedersen & Colmer, 2012). The surface of rice leaves is hydrophobic and the leaves initially retain gas films when submerged (Supporting Information Fig. S1), but the gas layer can diminish with time under water and result in reduced photosynthesis (Winkel et al., 2014) and a lower O2 status (Winkel et al., 2013). Research on the physiological roles of leaf gas films has previously relied on experimental removal of surface hydrophobicity using a dilute detergent so that gas films were not formed during submergence (Raskin & Kende, 1983; Colmer & Pedersen, 2008; Pedersen et al., 2009). However, a molecular genetic approach using a rice mutant with diminished capacity to retain leaf gas films will enable elucidation of the genetic regulation of this leaf trait and improve knowledge of the physiological function in plant submergence tolerance.

Leaf hydrophobicity has been studied in detail for some species (e.g. sacred lotus; Barthlott & Neinhuis, 1997) and it occurs as a result of various macro-, micro- and nanostructures on the surface – plicate leaves, papillae and epicuticular waxes, respectively (Marmur, 2003; Koch & Barthlott, 2009) – although the epicuticular wax platelets are considered to be of particular importance (Koch & Barthlott, 2009; Herzog et al., 2017). Synthesis of epicuticular waxes involves fatty acyl-CoA elongation, the products of which are then catalysed to primary alcohols by fatty acyl reductases, a process that in Arabidopsis is controlled by members of the CER gene family (Samuels et al., 2008). ECERIFERUM4 (CER4) encodes an alcohol-forming fatty acyl-coenzyme A reductase involved in epicuticular wax production in Arabidopsis, specifically in conversion of C26 and C28 very long-chain fatty acyl-CoA to the respective (same C number) primary alcohols (Rowland et al., 2006). In rice, OsHSD1 encodes a hydroxysteroid dehydrogenase (HSD) that influences leaf wax composition, which was suggested to occur via interactive effects of OsHSD1 on expression of CER genes (Zhang et al., 2016). Leaf surface hydrophobicity was lost in an oshsd1 mutant (Zhang et al., 2016), but the influence on leaf gas films, underwater photosynthesis and submergence tolerance was not evaluated. The importance of leaf hydrophobicity and gas films for underwater photosynthesis and submergence tolerance of rice (Pedersen et al., 2009; Winkel et al., 2013; Colmer et al., 2014) and some other species (Colmer & Pedersen, 2008; Colmer et al., 2011) prompted the present study, which aimed to identify a gene conferring leaf gas film retention in rice and its phenotypic influence.

The present study used a rice mutant (dripping wet leaf 7, dpr7) with leaves that were significantly less hydrophobic than the wild-type (Satoh et al., 1983), evident as water adhesion rather than water shedding during rainfall. We tested the hypothesis that upon submergence the dpr7 mutant does not retain leaf gas films and elucidated the greatly reduced gas film retention phenotype of the dpr7 mutant, followed by gene mapping and a gene complementation test. Discovery of the gene determining leaf gas film retention, Leaf Gas Film 1 (LGF1), demonstrated that LGF1 determines leaf wax composition, epicuticular wax platelet abundance, surface hydrophobicity, and thus gas film retention and underwater photosynthesis for leaves of rice.

Materials and Methods

Plant material

Rice (Oryza sativa L.) cv Kinmaze and the dpr7 mutant (Satoh et al., 1983) from a N-methyl-N-nitrourea mutagenized population were provided by the Laboratory of Plant Breeding, Kyushu University. Other rice materials used to generate a mapping population and to make transgenic plants for a gene complementation test are described in the relevant sections later in the paper. Plants were grown in pots of flooded rice paddy soil in trays of deionized water either in a glasshouse (June-to-August, Nagoya, Japan) for the mapping population and the transgenic plants, or in a controlled-environment room (30 : 25°C, light : dark periods; 16 h light period of 100 μmol m−2 s−1 photosynthetically active radiation (PAR)) for the characterization of the leaves of Kinmaze and the dpr7 mutant.

Leaf hydrophobicity and gas film thickness

Hydrophobicity of the leaf cuticle was assessed using the contact angle of small droplets of water, and contact angles > 90° indicate a hydrophobic surface and those > 150° indicate superhydrophobicity (Koch & Barthlott, 2009). Hydrophobicity of leaf blade segments of Kinmaze, dpr7, pUb(KC) and pUb::LGF1 was quantified by measuring the contact angle of a 1 μl water droplet on the adaxial side with a wettability measurement system (LSE-ME3; Nick, Kanayamacho12-1, Kawaguchishi, Japan). The contact angle of each sample was calculated by averaging the values of three measurements for 5 s. Samples were from the youngest fully expanded leaf of rice at the seventh leaf stage.

Gas film thickness on submerged leaf blade segments was measured using the buoyancy method (Raskin, 1983; Colmer & Pedersen, 2008). The buoyancy of leaf segments (c. 5 cm in length) taken around one-third of the distance from the tip of the youngest fully expanded leaf was measured in deionized water with the sample mounted on a hook underneath a four-digit balance with measurements before and after brushing both surfaces of the leaf segment with 0.1% (v/v) Triton X-100 to remove any gas films. Leaf gas film thickness was calculated as: gas volume (m³)/[2 x projected area (m²)] (see Colmer & Pedersen (2008) for details).

Leaf net photosynthesis: under water and in air

Underwater net photosynthesis was assessed by measuring O2 evolution in a closed system according to the method of Pedersen et al. (2013). In brief, leaf blade segments (c. 2.5 cm in length) taken around one-third of the distance from the tip of the youngest fully expanded leaf were inserted into glass bottles which contained 25 ml of medium, and two glass beads were added to ensure mixing as the bottles rotated inside the illuminated water bath at 30°C; one leaf segment was placed in each
bottle. The PAR inside the glass bottles was 1000 μmol m$^{-2}$ s$^{-1}$ (measured using a spherical sensor; 4p US-SQS/L; Walz, Effeltrich, Germany). The incubation medium was based on the general purpose culture medium described by Smart & Barko (1985) and contained (in mmol m$^{-3}$): Ca$^{2+}$, 0.62; Mg$^{2+}$, 0.28; Cl$^{-}$, 1.24; SO$_4^{2-}$, 0.28. Various amounts of KHCO$_3$ were added to the incubation medium, and 0.1 M HCl was used to adjust the pH (6.3–8.7), thus converting HCO$_3^{-}$ into free CO$_2$ to achieve a range of dissolved CO$_2$ (0.5–2500 mmol m$^{-3}$), whilst keeping HCO$_3^{-}$ constant at 2.0 mol m$^{-3}$. The dissolved O$_2$ concentration in the incubation medium was set at c. 50% of air equilibrium applied to prevent an increase in O$_2$ above air equilibrium levels during measurements that might have led to photorespiration and thus decreased net photosynthesis (Colmer & Pedersen, 2008). Following incubations of known duration, the dissolved O$_2$ concentration in each bottle was measured using a calibrated O$_2$ mini-electrode (OX-500; Unisense A/S, Aarhus, Denmark) connected to a pico-amperemeter (PA2000; Unisense A/S). Dissolved O$_2$ concentrations in bottles prepared and incubated in the same way, but without leaf segments, served as blanks. The projected area of each leaf segment was measured using digital photography followed by analysis with IMAGEJ software (Schneider et al., 2012).

Rates of net photosynthesis in air were measured about one-third of the distance from the tip of the youngest fully expanded leaf blade on the main stem of rice. A Li-Cor 6400XT (Li-Cor, Lincoln, NE, USA) photosynthesis system was used with an external light source of 1000 μmol m$^{-2}$ s$^{-1}$ PAR, block temperature of 28°C, flow rate of 500 μmol s$^{-1}$, CO$_2$ of 800 μmol mol$^{-1}$ (twice atmospheric concentrations to overcome any stomatal limitations on CO$_2$ entry, as the aim was to evaluate the capacity of the photosynthetic machinery in air for benchmarking against maximal rates when under water), and relative humidity (RH) of 80%. In some experiments net photosynthesis in air was measured with ambient CO$_2$ at 400 μmol mol$^{-1}$ and 46% RH (Kinmaze and drp7 mutant) or 80% RH (complementation lines and vector controls), with flow rate, block temperature and PAR as before.

Scanning electron microscopy of leaf surfaces

Surface structures (papillae and epicuticular wax platelets) on the adaxial surface of leaf blades were visualized using a scanning electron microscope. Samples were taken at around one-third of the distance from the tip of the youngest fully expanded leaf blade of rice at two growth stages (see relevant figure captions) and were gold-coated by Smart Coater (Jeol, Musashino, Akishimashi, Tokyo, Japan) for 2 min and viewed with a scanning electron microscope (JCM-6000: Jeol), operating at an accelerating voltage of 15 kV under high vacuum mode.

Identification of LGF1: gene mapping, transcript abundance, and complementation test

In order to map the LGF1 gene, F$_2$ plants of crosses between the drp7 mutant (japonica) and Kasalath (indica) were used. A total of 5300 individual plants of the F$_2$ population were used in positional cloning of LGF1. Genomic DNA was extracted from each plant of the F$_2$ population using the modified TPS method (Thomson & Henry, 1995; Nagai et al., 2012). The purified DNA samples were then genotyped using molecular markers. PCR-based markers, including simple sequence repeat markers (https://www.ricebase.org), cleaved amplified polymorphic sequence (CAPS) markers (for this study, CAPS markers were designed using dCAPS FINDER 2.0 and single nucleotide polymorphisms, which were identified by comparing the genomic DNA sequences of each parent, were used for mapping.

To confirm the coding sequences of each gene in the candidate region, we designed the sequence primers (Table S1) and amplified the genomic regions using genomic DNA from the drp7 mutant. Amplified DNA fragments were electrophoresed and purified using Wizard SV Gel and PCR Clean-up System Kit (Promega). Sequencing of DNA fragments was performed using an ABI3730xl capillary DNA sequencer (Applied Biosystems, Waltham, MA, USA). Sequences of candidate genes were compared with annotated sequences on RAP-DB (http://rapdb.dna.affrc.go.jp) and TIGR (http://rice.plantbiology.msu.edu) using ATGQ software (Genetyx, Tokyo, Japan).

Total RNA was isolated from roots, youngest fully expanded leaf sheath and leaf blade and expanding leaf blade of Kinmaze and the drp7 mutant grown in a glasshouse for 2 months, using the RNeasy Plant Mini Kit (Qiagen). First-strand cDNA was generated using Omniscript RT kit (Qiagen). Real-time quantitative PCR analysis was carried out using StepOnePlus (Thermo Fisher, Waltham, MA, USA). The cDNAs of interest were specifically amplified with the following primers: LGF1-RT-F (5’-TCACGCAAGAGATCCTCGAG) and LGF1-RT-R (5’-CATGGCTGGGCGGATGGTCTTG). As a control for quantitative reverse transcription polymerase chain reaction, Ubiquitin was amplified with the primers Ubi-F (5’-AATTCCAATCTTCTTGCCTC) and Ubi-R (5’-TGTCAATCGTATCGGAGAAC). Each 20 μl reaction mixture contained 1 × SYBR Fast qPCR Mix, 0.4 μM gene-specific primers and 1 × ROX reference dye. Reactions were performed according to the manufacturer’s instructions. The relative mRNA expression levels were normalized against Ubiquitin gene expression levels.

The full-length coding sequence of LGF1 (OsHSD1 (LOC_Os11g30560)) was amplified using cDNA. The primers were designed on the basis of the information on TIGR (http://rice.plantbiology.msu.edu). The coding sequence was ligated into pCAMBIA1380 containing rice Ubiquitin promoter using a DNA ligation kit (Takara, Shimgyo-ku, Kyoto, Japan), following the manufacturer’s protocol. The construction was transformed into Agrobacterium tumefaciens strain EHA105 by electroporation (Hood et al., 1986). Generation of transgenic rice plants used Agrobacterium-mediated transformation of rice calli according to the method described by Hiei et al. (1994). The transformed plants (T0 plants) were used in experiments.

Leaf cuticular wax composition

Total cuticular wax mixtures were extracted by immersing leaf blades in chloroform for 60 s at 60°C (Zhou et al., 2013). The
solution was completely evaporated under a stream of N₂ gas, and the residue was redissolved in chloroform. Wax identification and quantification were performed using published methods with minor modifications (Greer et al., 2007). In thin-layer chromatography analyses, the total wax was separated on silica gel with hexane/diethyl ether/acetate (90:7.5:1, v/v/v) and each wax compound was scraped off from the silica gel after being visualized by staining with primuline and UV light. n-Tetrasosane (C24 alkane) was added to the silica gel as an internal standard, and extracted with chloroform. The solution was evaporated under a stream of N₂ gas, and the residues were incubated with N,O-bis(trimethylsilyl) trifluoracetamide with 1% trimethylchlorosilane and pyridine for 1 h at 80°C. BSTFA and pyridine were then removed under a stream of N₂ gas. The samples were dissolved in hexane and analysed for wax component identification by GC-MS (GCMSTQ-8030; Shimadzu, Nakagyouku, Kyoto, Japan), equipped with an Rxi-5HT column (length, 30 m; internal diameter, 0.25 mm; film, 0.25 μm; Restek, Benner Circle, Bellefonte, PA, USA), and for wax quantification by GC-flame ionization detection (GC-FID) (GC2014; Shimadzu), equipped with a DB-5 column (length, 30 m; internal diameter, 0.25 mm; film, 0.25 μm; Agilent, Santa Clara, CA, USA). For the GC-MS and GC-FID, the carrier gas was He, the injection port temperature was as follows: 50°C for 2 min, increased to 200°C at a rate of 40°C min⁻¹ and held for 2 min, after which it was raised to 320°C at a rate of 3°C min⁻¹ and held for 40 min. Wax quantities were determined by comparing FID peak areas against internal standards. The data were expressed per unit surface area extracted for each sample.

Sequence data
The sequence data of LGF1 and lgf1 have been deposited in the EMBL/GenBank/DBJ Nucleotide Sequence Databases under the accession numbers LC363889 and LC363890, respectively.

Data analyses
Graphpad PRISM 7.0 (La Jolla, CA, USA) was used for statistical analyses and graphing of data. Figure and table captions provide details on the various tests used and significance levels.

Results
Leaf gas films are soon lost from the drp7 mutant when submerged and underwater photosynthesis declines
We investigated leaf gas film retention upon submergence, and leaf surface hydrophobicity for Kinmaze and the drp7 mutant. Both genotypes possessed leaf gas films when first submerged, but the gas films on the drp7 mutant did not persist beyond 1 d of submergence (Fig. 1a,b). Loss of the gas films was associated with a loss of leaf surface hydrophobicity (Fig. 1c–f). Water droplets were repelled from leaves of Kinmaze after 1 d of submergence, but not from the drp7 mutant. Leaf surface hydrophobicity was quantified by contact angle measurements which showed that the drp7 mutant had become hydrophilic after 1 d of submergence whereas Kinmaze remained hydrophobic (Fig. 1c–f). Interestingly, leaves of Kinmaze possess a higher density of epicuticular wax platelets than those of the drp7 mutant (Figs 1g,h, S2), and these wax platelets contribute to leaf hydrophobicity (Neinhuis & Barthlott, 1997; Koch & Barthlott, 2009). Papillae density can also influence leaf hydrophobicity (Koch et al., 2009), but the densities of the drp7 mutant and Kinmaze were similar (Fig. 1g,h).

The gas film thickness on submerged leaves was maintained for Kinmaze over 4 d of submergence, whereas the drp7 mutant did not retain gas films after 1 d of submergence (Fig. 2a). Loss of gas films caused a marked reduction in net photosynthesis of submerged leaves of the drp7 mutant, whereas this was maintained for Kinmaze (Fig. 2b). CO₂ response curves for underwater net photosynthesis were measured for leaves after 1 d of plant submergence and showed substantially higher rates for Kinmaze than for the drp7 mutant (Fig. 2c). Underwater net photosynthesis of Kinmaze was CO₂-saturated at 200 μmol m⁻² s⁻¹ in the water, whereas the drp7 mutant required c. 10-fold higher external dissolved CO₂. Indeed, the resistance to CO₂ entry was 8.2-fold higher for the drp7 mutant (gas films absent) than for Kinmaze (gas films present) with 12–200 μmol m⁻² s⁻¹ in the water (Fig. 2c). By contrast with the situation when submerged, capacity for net photosynthesis in air did not differ between the two genotypes (Fig. S3a). Leaf blade Chl content (soil plant analysis development, readings) and tissue thickness also did not differ between the two genotypes (Fig. S3b,c).

Identification of a gene determining leaf hydrophobicity and gas film retention
To identify the gene responsible for the drp7 mutant phenotype of leaf gas film loss, we crossed the drp7 mutant with Kasalath (O. sativa Indica), and scored the F₁ and F₂ progenies for the dripping wet leaf phenotype. All F₁ plants were water-repellent and for the F₂ population of 5300 individuals the segregation ratio was 3:1 for water-repellent, dripping wet leaf phenotypes, indicating a recessive mutation of a single gene, according to Mendelian inheritance. We used positional cloning with the 5300 F₂ plants to identify a candidate region of 221 kb on chromosome 11, between markers RM26764 and RM26774 (Fig. 3a). Further high-resolution mapping using dCAPS and sequencing reduced the candidate region to 154 kb (Fig. 3b), which contained 18 annotated genes in TIGR (http://rice.plantbiology.msu.edu) (Fig. 3c). Genomic DNA sequence analysis indicated a single nucleotide substitution in LOC_Os11g30560.1 (Fig. 3d) of an ‘A’ in Kinmaze to a ‘T’ in the drp7 mutant at the 3’-splicing site in the third intron of the OsHSD1 gene, which is a member of the short-chain dehydrogenase reductase (SDR) family (Fig. 3e). Moreover, cDNA sequence analysis indicated that this single nucleotide substitution resulted in a premature stop codon in OsHSD1 of the drp7 mutant, as well as a 29 bp upstream extension of the fourth exon (Fig. 3e). This premature stop codon would presumably yield a truncated protein product (Fig. S4), possibly resulting in loss of the predicted catalytic
residues and thus loss of function in the mutant (i.e. loss of leaf gas films).

The present mutation differs from those reported for other rice wax mutants (Rowland et al., 2006; Yu et al., 2008; Islam et al., 2009; Park et al., 2010; Chen et al., 2011; Qin et al., 2011; Mao et al., 2012; Zhou et al., 2013; Zhu & Xiong, 2013) and is a different allele of OsHSD1 to that recently identified by Zhang et al. (2016) to encode an HSD involved in wax synthesis. The importance of leaf gas films for submergence tolerance of rice (Pedersen et al., 2009) and the loss of capacity for gas film retention in the drp7 mutant (Figs 1b, 2a) prompted us to name the new allele discovered here Leaf Gas Film 1 (LGF1). Consistent with its role in determining leaf cuticle wax composition, LGF1 was preferentially expressed in the expanding leaf blade of Kinmaze (Figs 3f, S5). By contrast, the drp7 mutant showed little expression of LGF1. This result suggests that the drp7 mutant is a null mutant of LGF1.

Verification of the function of LGF1/OsHSD1 by a complementation test

The function of LGF1 was verified with a complementation test by generating transgenic lines of rice with LGF1 cDNA from Kinmaze driven by the Ubiquitin-promotor in the drp7 mutant (pUb::LGF1/drp7), as well as vector control lines in the drp7 mutant (pUb(VC)/drp7). T0 plants were raised in seedling trays in a glasshouse and tested for the dripping wet leaf phenotype as described earlier for the mapping population. T0 plants displaying the nondripping wet leaf characteristic (i.e. with water-repellent leaves), as well as a random selection of T0 vector control lines, were sampled for further measurements as described later. Gas film retention (Fig. 4a,b), leaf surface hydrophobicity (Fig. 4c–f) and density of epicuticular wax platelets (Fig. 4g,h) were all restored in the complementation lines expressing LGF1, but not in the vector control lines. Moreover, thickness of the gas film (Fig. 5a) and the rate of underwater net photosynthesis (Fig. 5b) were both also restored in the LGF1 complementation lines, as compared with the vector control lines. By contrast with the enhanced photosynthesis when under water, when in air the leaves of the two lines did not differ in net photosynthesis (Fig. S6).

LGF1/OsHSD1 influences leaf wax chemistry

Leaf wax components were evaluated using GC-MS (Fig. S7) and GC-FID (Figs 6a,b and S7). The drp7 mutant contained...
whereas underwater very high, because then enough CO2 can diffuse into both leaf types for of the response curve (i.e. when CO2 concentrations were low) shows that whereas for the underwater P

ments of younger plants (Fig. S8). Importantly, no other wax

significantly less C30 primary alcohol, but more C30 aldehyde, than did Kinmaze (Fig. 6a), which was also verified by measurements of younger plants (Fig. S8). Importantly, no other wax components differed between the drp7 mutant and Kinmaze (Figs 6a, S8). The pUc::LGF1::drp7 complementation lines, when compared with its vector control lines, showed more C30 primary alcohol and relatively low amounts of C30 aldehyde (Fig. 6b). These wax composition results (Fig. 6), together with the observations of epicuticular wax platelet abundances (Figs 1g, h, 4g,h), provide evidence that LGF1 determines the balance between C30 primary alcohol and C30 aldehyde and that the C30 primary alcohol is required for formation of abundant epicuticular wax platelets, increasing leaf surface hydrophobicity and gas film retention during submergence.

Additional characterization of the drp7 mutant

The importance of leaf hydrophobicity for paddy rice was illustrated by the death of the drp7 mutant within 3 wk after transplantation into c. 15 cm of standing water in a field during the wet season in Japan, whereas Kinmaze remained green and continued to grow (Fig. S9a–c). The leaves of the drp7 mutant could not maintain their emergence above water and eventually died, whereas leaves of Kinmaze maintained air contact and the plants continued to grow. As cuticle properties also influence water loss and pathogen infections (Samuels et al., 2008), we also tested the responses of the drp7 mutant to water deficit and to rice blast disease. The drp7 mutant showed sensitivity to water deficit (Fig. S10a,b), associated with faster leaf water loss (Fig. S10c) and lower net photosynthesis in air of low humidity (46% RH), compared with Kinmaze (Fig. S10d). The drp7 mutant was more susceptible than Kinmaze to infection by rice blast (Fig. S11). These results show the wider significance of LGF1 for stresses beyond its role in leaf gas film retention and tolerance of complete submergence.

Discussion

Hydrophobic leaves with gas film retention are of importance for rice crops. Our study identified LGF1/OsHSD1, the gene determining this leaf trait. Hydrophobic body surfaces that retain a thin layer of gas when submerged are a mechanism to enhance gas exchange with water, evident both in aquatic invertebrate animals and in some plants (Thorpe & Crisp, 1947; Hebets & Chapman, 2000; Pedersen & Colmer, 2012). However, the molecular genetic mechanism of leaf gas film retention had not been elucidated. As discussed in the following, the LGF1 allele of OsHSD1 determines the amount of C30 primary alcohol and therefore the C30 primary alcohol-to-aldehyde ratio, which is of importance for formation of abundant epicuticular wax platelets on leaves of rice, which in turn strongly affects surface hydrophobicity, gas film retention, and underwater photosynthesis during submergence.

Most interestingly, LGF1/OsHSD1 is not a CER-like gene, of which seven are known for rice (Fig. S12). The influence of OsHSD1 on leaf wax composition has been suggested to occur via interactive effects of OsHSD1 on expression of CER genes (Zhang et al., 2016). LGF1/OsHSD1 encodes an HSD, being one member of an HSD gene subfamily (Fig. S12; Zhang et al., 2016). LGF1 and OsHSD1 encode the same gene; however, the two mutant alleles differ for the drp7 (present study) and the
oshsd1 (Zhang et al., 2016) mutants. Moreover, the leaf wax composition of the drp7 mutant showed specific changes in the levels of only two C30 wax components (verified also for the LGF1 complementation lines in the drp7 background), whereas the oshsd1 mutant showed changes in the levels of several other wax components (Zhang et al., 2016). The differences in wax composition between the two mutants could be a result of the substantial differences in the mutation events: the predicted truncated protein in the drp7 mutant (stop codon in the fourth exon) vs the larger predicted protein as a result of an amino acid deletion and an amino acid substitution in the oshsd1 mutant. In addition, the expression level of LGF1 in the drp7 mutant was substantially lower in various tissues than in Kinmaze (Fig. 3f), indicating that the drp7 mutant has a putative null allele. Thus, the drp7 mutant differs markedly from the oshsd1 mutant, which showed lower expression of OsHSD1 but had the same enzymatic activity as its wild-type (Zhang et al., 2016). Moreover, although leaf hydrophobicity and wax platelet abundance were studied for the oshsd1 mutant (Zhang et al., 2016), gas film retention was not evaluated, so the present data on the drp7 mutant extend our knowledge of the LGF1/OsHSD1 gene and also provide a comprehensive phenotypic analysis of these leaf surface properties.

Fig. 3 Identification of Leaf Gas Film 1 (LGF1) which determines leaf gas film retention in rice. (a) The gene associated with gas film loss in the drp7 mutant was rough-mapped to the long-arm of chromosome 11. (b-d) The physical map of the candidate region on chromosome 11 (numbers above the red arrow indicate number of recombinants) (b) and the candidate region (c) contains 18 annotated genes (TIGR), with the candidate gene identified as OsHSD1 by genomic DNA sequence comparison of Kinmaze and the drp7 mutant (d). (e) Gene structure and sequence showing the mutation site at the 3'-splicing site in the third intron of OsHSD1 in the drp7 mutant (single nucleotide polymorphism of 'T' in drp7 vs 'A' in Kinmaze) resulted in a stop codon (indicated with *) as well as a 29 bp upstream extension of the fourth exon. The resulting truncated mRNA presumably explains the loss-of-function mutation (i.e. loss of leaf gas films) and so we named this allele Leaf Gas Film 1 (LGF1). (f) The expression level (transcript abundance) of LGF1 in roots, youngest fully expanded leaf sheath and leaf blade, and expanding leaf blade of Kinmaze and the drp7 mutant (see Supporting Information Fig. S5 for diagram showing these organs). Values are means ± SD (n = 3), with each replicate being from a different plant. Ubiquitin (LOC_Os04g53620) was used as an internal control for normalization. Expression level in the expanding leaf blade of Kinmaze was used for reference expression level. See Table S1 for data on primers.
Fig. 4 Complementation test of LGF1 expressed in the drp7 mutant background. (a, b) Leaf gas films were not retained on leaves of the vector control lines pUb(VC) in the drp7 background (a), whereas gas film persistence was restored for the pUb::LGF1 complementation lines (Kimmaze LGF1 in drp7 background) as evident after 1 d of submergence (b). When present, gas films are evident as a silvery sheen on the lower half of leaves dipped into water; the white triangle shows the water level. (c, d) The leaf blade surface is initially hydrophobic on the vector control pUb(VC) lines and the pUb::LGF1 complementation lines, as demonstrated by the water droplet test. (e, f) Within 1 d of plant submergence, the leaf surface of the vector control pUb(VC) lines had become hydrophilic, but leaves remained hydrophobic for the pUb::LGF1 complementation lines. (c–f) Contact angles of water droplet and leaf surface were measured, as indicated by the dotted red lines. (g, h) Scanning electron micrographs of leaf blades showed a relatively low density of epicuticular wax platelets for the vector control pUb(VC) line, but that epicuticular wax platelet density had increased for the pUb::LGF1 complementation lines. The numerous, small white ‘flecks’ on the leaf surface are the epicuticular wax platelets, whereas the larger circular-like structures (top view of a dome-like protrusion) are papillae (labelled ‘p’). All measurements were on the adaxial side of leaf 7 of first-generation (T0) plants (each replicate used an individual T0 plant from an independent transformation event). Contact angles are of a 1 µm water droplet (side views in the figure; the top views used larger drops of water) and are the angles (relative to the horizontal) at which the water–air interface of the droplet meets the leaf surface. Data in (c)–(f) are means ± SD (n = 4) with each replicate being a leaf blade segment from a different plant. The greater mean contact angle of water droplets on the pUb::LGF1 complementation lines was significant (P < 0.0001; t-test) when compared with the vector control pUb(VC) line.

Fig. 5 Complementation test of LGF1 expressed in the drp7 mutant background. (a) Leaf gas film persistence was restored for the pUb::LGF1 complementation lines (Kimmaze LGF1 in drp7 background) as demonstrated by measurements of gas film thickness after 1 d of submergence. (b) Restoration of leaf gas films had a positive effect on underwater net photosynthesis ($P_N$) by the pUb::LGF1 complementation lines as compared with the vector control pUb(VC) lines (drp7 background). Measurements were on the youngest fully expanded leaf of first-generation (T0) plants (each replicate used an individual T0 plant from an independent transformation event). Data are means ± SD (n = 4 in both (a) and (b)) with each replicate being a leaf segment from a different plant. Significant difference (t-test): ****, $P < 0.0001$.

The finding that LGF1 specifically controls abundance of C30 primary alcohol (Figs 6a,b, S8) is a new insight into wax biosynthesis in rice. The specific increases in C30 aldehyde abundance and decrease in C30 primary alcohol for the drp7 mutant, and the recovery back to the wild-type levels in the LGF1 complementation lines (Figs 6a,b, S8), lead us to propose two hypotheses regarding the role of the LGF1 protein based on current knowledge of wax biosynthesis (Samuels et al., 2008), the relevant components of which are summarized in Fig. 7. The LGF1 protein is hypothesized to be involved in conversion of C30 aldehyde to C30 primary alcohol (Hypothesis 1) or of C30 very long-chain fatty acyl-CoA to C30 primary alcohol (Hypothesis 2). In the case of Hypothesis 1, if there is conversion of C30 aldehyde to the C30 primary alcohol (for this possibility in other organisms, see Kunst & Samuels (2003) and Zhou et al. (2014)) then inhibition of this reaction would result in accumulation of the C30 aldehyde and a deficit of the C30 primary alcohol. In the case of Hypothesis 2, if conversion of the C30 very long-chain fatty acyl-CoA directly to the primary alcohol is diminished (for this conversion in other organisms, see Samuels et al. (2008) and Wang et al. (2015)) then the flux along the alternative
pathway to C30 aldehyde would predominate. Thus, either of these two cases is consistent with the observed changes in the C30 wax components as found here for the *drp7* mutant and the *LGF1* complementation lines (Figs 6a,b, S8).

In addition to the influence of epicuticular wax platelets on leaf hydrophobicity (Marmur, 2003; Koch & Barthlott, 2009), papillae also contribute to leaf hydrophobicity (Koch & Barthlott, 2009), but this feature did not differ between the *drp7* mutant and Kinmaze (Figs 1g,h, S2). Development of papillae on rice leaves is regulated by *OsRopGEF10* (Yoo et al., 2011). Our observation of gas films initially being present on leaves of the *drp7* mutant upon submergence, but disappearing during the first day under water, might be explained by the papillae providing initial leaf hydrophobicity, with subsequent ingress of water onto the leaf surface layer because of the low abundance of epicuticular wax platelets.

The identification of *LGF1/OsHSD1*, a gene controlling leaf gas film retention when under water, adds to existing knowledge of the genetic basis of submergence tolerance in rice (e.g. Sub1A; Xu et al., 2006). Leaf gas films contribute to plant submergence tolerance as the resistance is lower for inward diffusion of CO2 and O2 into the leaves (Verboven et al., 2014), which aids photosynthesis and internal aeration during complete submergence (Pedersen et al., 2009; Winkel et al., 2013, 2016). The adaxial and abaxial surfaces of rice leaves are similarly hydrophobic and both surfaces possess gas films when submerged (Pedersen et al., 2009); the *drp7* mutant lost gas films from both leaf sides soon after submergence and gas film retention was restored on both sides for leaves of the *LGF1/OsHSD1* complementation lines.

The CO2 response curve of underwater net photosynthesis by Kinmaze and the *drp7* mutant at 1 d after submergence showed that at low external concentrations, the rate of CO2 entry into leaves was 8.2-fold higher for Kinmaze than for the *drp7* mutant (Fig. 2c). This 8.2-fold difference between leaves of Kinmaze (gas films present) and the *drp7* mutant (gas films absent) compares with the three- to fivefold higher CO2 entry into leaves with gas films present as compared with those for which gas films had been experimentally removed using a dilute detergent (Pedersen et al., 2009; Winkel et al., 2013). Importantly, when under water with 100 mmol CO2 m⁻³ (a concentration relevant in field situations; Colmer et al., 2011) the leaves of Kinmaze had a 4.5-fold higher rate of net photosynthesis compared with those of the *drp7* mutant (Fig. 2c), and the *LGF1/OsHSD1* complementation lines in the *drp7* background had a 2.7-fold higher rate of underwater net photosynthesis compared with those of the vector control lines (Fig. 5b). The present study of the *drp7* mutant and the *LGF1/OsHSD1* complementation lines, together with previous work which experimentally manipulated leaf gas films (Pedersen et al., 2009; Winkel et al., 2013) or modelled the function of this leaf trait (Verboven et al., 2014), demonstrates the significance of leaf gas film retention for underwater photosynthesis and submergence tolerance of rice.

In conclusion, *LGF1/OsHSD1* controls C30 primary alcohol synthesis, and the resulting C30 primary alcohol-to-aldehyde ratio influences the abundance of epicuticular wax platelets, which determines leaf hydrophobicity and confers retention of gas films on submerged leaves. This trait is essential for survival of rice even in paddy field conditions and contributes to...
submergence tolerance. Discovery of LGF1 provides an opportunity in the future to better understand variation among rice genotypes of leaf gas film retention (e.g. Winkel et al., 2014) and possibly to identify alleles that confer higher LGF1/OsHSD1 expression (and protein abundance) and/or higher activity of the enzyme, which could be related to epicuticular wax platelet abundance and the duration of gas film retention on leaves during submergence. If any allelic variation in LGF1/OsHSD1, either natural or induced, is associated with leaf gas film retention, this could be a future breeding target in addition to Sub1 (Ismail et al., 2013) for further improvement of rice submergence tolerance and yield stability of rice crops in flood-prone areas.

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References


**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information tab for this article:

**Fig. S1** Photographs and conceptual models of rice leaves with or without leaf gas films.

**Fig. S2** Leaf blade surface structure of Kinmaze and the *drp7* mutant.

**Fig. S3** Net photosynthesis in air, Chl content (SPAD reading) and leaf thickness of Kinmaze and the *drp7* mutant.

**Fig. S4** Sequence comparison of OsHSD1 between Kinmaze and the *drp7* mutant.

**Fig. S5** Schematic diagram of a rice plant showing the organs from which RNA was extracted to assess *LGF1* expression (transcript abundances).

**Fig. S6** Net photosynthesis in air by the *pUb*: *LGF1* complementation lines (Kinmaze *LGF1* in *drp7* background) and the vector control *pUb*(VC) lines (*drp7* background).

**Fig. S7** Main components of leaf waxes (TLC and GC-MS) of Kinmaze and the *drp7* mutant.

**Fig. S8** Wax composition (GC-FID) for leaf blades of Kinmaze and the *drp7* mutant.

**Fig. S9** Evaluation of Kinmaze and the *drp7* mutant in a flooded paddy field.

**Fig. S10** Responses to water deficit of Kinmaze and the *drp7* mutant.

**Fig. S11** Susceptibility to rice blast disease for Kinmaze and the *drp7* mutant.

**Fig. S12** Phylogenetic analysis of SDR (FAR) and HSD gene families in Arabidopsis and rice.

**Table S1** Primers used in this study

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