Improvement of water use efficiency in rice by expression of *HARDY*, an *Arabidopsis* drought and salt tolerance gene

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Freshwater is a limited and dwindling global resource; therefore, efficient water use is required for food crops that have high water demands, such as rice, or for the production of sustainable energy biomass. We show here that expression of the *Arabidopsis HARDY* (*HRD*) gene in rice improves water use efficiency, the ratio of biomass produced to the water used, by enhancing photosynthetic assimilation and reducing transpiration. These drought-tolerant, low-water-consuming rice plants exhibit increased shoot biomass under well irrigated conditions and an adaptive increase in root biomass under drought stress. The *HRD* gene, an AP2/ERF-like transcription factor, identified by a gain-of-function *Arabidopsis* mutant *hrd-D* having roots with enhanced strength, branching, and cortical cells, exhibits drought resistance and salt tolerance, accompanied by an enhancement in the expression of abiotic stress associated genes. *HRD* overexpression in *Arabidopsis* produces thicker leaves with more chloroplast-bearing mesophyll cells, and in rice, there is an increase in leaf biomass and bundle sheath cells that probably contributes to the enhanced photosynthesis assimilation and efficiency. The results exemplify application of a gene identified from the model plant *Arabidopsis* for the improvement of water use efficiency coincident with drought resistance in the crop plant rice.

biomass | bundle sheath | photosynthesis | root strength | transcription factor

Water scarcity, caused by the rapidly increasing world population and the accompanying increases in water use for social and economic development, threatens sustainable world crop production that consumes most of the global water resources (1, 2). Rice uses two to three times more water than other food crops such as wheat or maize and uses 30% of the freshwater used for crops worldwide (3). Rice is also the primary source of food for more than half of the world's population, especially in developing countries in Asia, where water scarcity and drought are an imminent threat to food security (3, 4). Thus, a more sustainable use of global water resources in crop production is essential.

Water use efficiency (WUE), measured as the biomass produced per unit transpiration, describes the relationship between water use and crop production. In water-limiting conditions, it would be important to produce a high amount of biomass, which contributes to crop yield, using a low or limited amount of water. Environmentally sustainable biomass production in bioenergy crops (5) would also reduce competition for land use and limited water resources with food crops required to feed the growing population.

The basic physiological definition of WUE equates to the ratio of photosynthesis (A) to transpiration (T), also referred to as transpiration efficiency. Although genetic variation for WUE has been observed in crop plants (6), its molecular dissection has only recently been initiated in the model *Arabidopsis*, where the *ERECTA* gene was found to be critical in altering transpiration efficiency (7) by mechanisms including leaf epidermal and mesophyll differentiation. However, so far, the engineering of major field crops for improved WUE with single genes has not yet been achieved.

Water scarcity can impose abiotic stresses like drought and salinity, which are among the most important factors limiting plant performance and yield worldwide (8). Plant resistance to drought stress can be improved through drought avoidance or drought tolerance (9), among which drought avoidance mechanisms tend to conserve water by promoting WUE. The concomitant improvement in WUE and drought stress resistance without yield penalties can offer long-term sustainable solutions to agricultural land use. In this report, we provide a way to achieve improved WUE and drought resistance in rice, the crop that feeds billions of humans in water-scarce areas of the world.

Results

Identification of the *HARDY* (*HRD*) Gene in *Arabidopsis*. In a phenotypic screen of an activation tagged mutant collection in *Arabidopsis* (10), a gain-of-function mutant *hardy* (*hrd-D*; "D" denotes the dominant effect) was identified with the remarkable feature of having roots that were difficult to pull out from the soil and dark green leaves that were smaller and thicker than normal WT plants (Fig. 1 *A* and *B*). Molecular characterization of the mutant revealed that the activation tag insert was adjacent to the *At2g36450* gene (Fig. 2*A*), an AP2/ERF-like transcription factor, that was overexpressed in the *hrd-D* mutant (Fig. 2*B*). A construct was made with the predicted coding region of the *At2g36450* gene under control of the CaMV35S promoter

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Abbreviations: CWT, cumulative water transpired; DAS, days after sowing; GO, Gene Ontology; LAD, leaf area duration; MTR, mean transpiration rate; NAR, net carbon assimilation rate; PSII, photosystem II; WUE, water use efficiency.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE8936).

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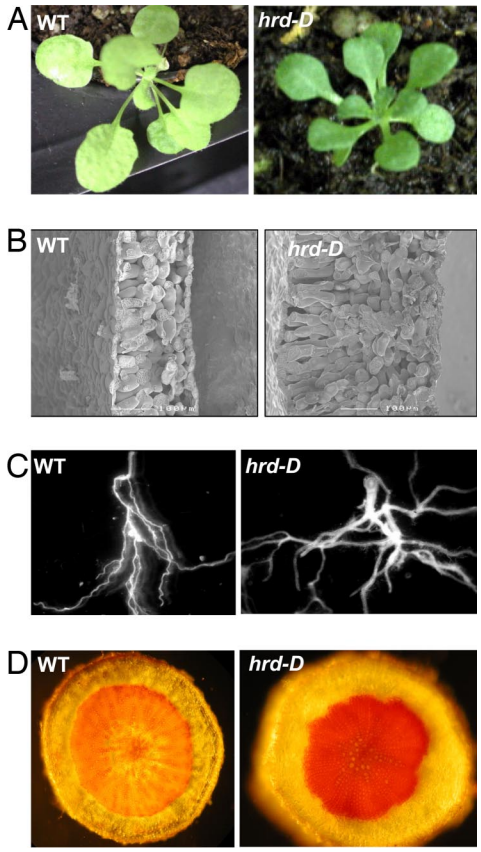

Fig. 1. The *hrd-D* mutant phenotype in *Arabidopsis*. (*A*) Rosette leaf phenotype of WT and *hrd-D* mutant with smaller, slightly curled, thicker deep-green leaves. (*B*) Cryo-fracture scanning electron microscopy section of leaves of WT and *hrd-D* mutant, showing more mesophyll cell layers. (*C*) Root structure of WT and *hrd-D* mutant, showing more profuse secondary and tertiary roots at the root base. (*D*) Cross-section of WT and *hrd-D* roots, showing increased cortical cell layers (lighter stained) and compact stele in the mutant.

[supporting information (SI) Fig. 6] and transformed into *Arabidopsis*. The transformants displayed the typical *hrd-D* phenotype (Fig. 1*A*) to various degrees of severity (data not shown).

To test whether the *HRD* gene could be induced to exhibit the different phenotypes, we generated transgenic lines that express a fusion protein of HRD and the hormone binding domain of the rat glucocorticoid receptor (GR) (11) under the control of the CaMV35S promoter (SI Fig. 6*C*). These HRD-GR lines could be induced with strong treatments (sprayed from top and watered from bottom) of the steroid hormone dexamethasone (DEX) to exhibit a typical *hrd-D*-like mutant phenotype of dark green leaves (see SI Fig. 7 and *SI Text*). On basis of these phenotype recapitulation experiments, we termed the predicted At2g36450 gene *HARDY* (*HRD*), responsible for the *hrd-D* mutant phenotype.

Phenotype of *HRD* Overexpression in *Arabidopsis*. Analysis of the *hrd-D* mutant leaf sections revealed extra palisade and spongy mesophyll layers compared with WT, contributing to the thicker leaf structure (Fig. 1*B*). The extended palisade layer bears abundant chloroplasts and contributes to the deeper green leaf color. The root phenotype was examined by growing *hrd-D* and WT plants ($n = 4$) in sand with nutrients for 4 weeks and then analyzing the number and length of the primary, secondary, and tertiary roots (SI Table 1). The distinguishing feature of the *hrd-D* mutant was the increased secondary and tertiary roots

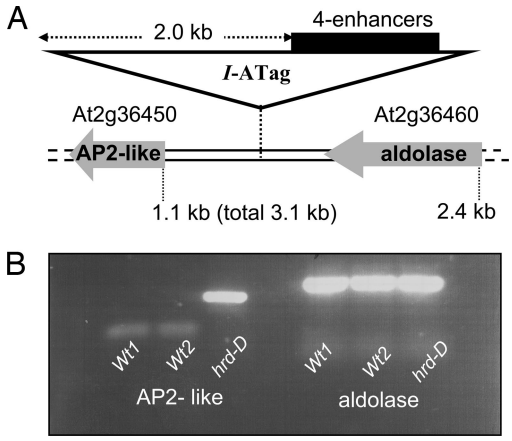

Fig. 2. The *hrd-D* mutant and expression analysis. (*A*) The *hrd-D* mutant genomic region, showing adjacent genes, annotated as AP2-like and aldolase, with their promoters located, respectively, 3.1 kb and 2.4 kb from the CaMV35S enhancer tetramer of the *I*-ATag transposon insert (10). (*B*) RT-PCR expression analysis of the AP2-like and aldolase genes, using RNA from rosette leaves of two WT (Wt1, Wt2) samples and the *hrd-D* mutant. The aldolase gene is highly expressed and unchanged, whereas the AP2-like gene is overexpressed in the *hrd-D* mutant, with the weak lower bands being primer–dimers.

along 1 cm of the root base compared with the WT, giving rise to a denser root network (Fig. 1*C*).

The strength of the *hrd-D* mutant roots was quantified by an assay to measure the root-pulling force for mature plants grown under well watered conditions, showing that the *hrd-D* mutant required 20–50% more force to pull it out from the ground compared with WT (SI Fig. 8). Cross-sections of the *hrd-D* root showed extra cortical cell layers and a more compact stele-bearing vascular tissue (Fig. 1*D*).

A test for drought resistance (12) showed that the *hrd-D* mutant survived longer periods of drought stress than WT plants (Fig. 3*A* and SI Fig. 9). In a salt-tolerance assay (13), the *hrd-D* mutant survived salt stress as high as 300 mM NaCl and could reach full maturity, in contrast to the WT (Fig. 3*B* and SI Table 2). In addition, the HRD-GR lines tested in an inducible

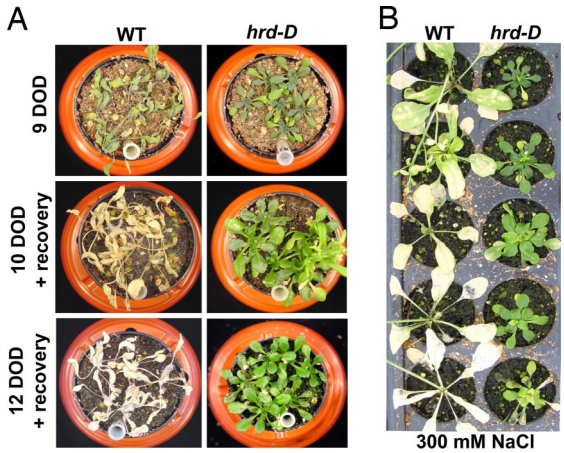

Fig. 3. Stress tolerance/resistance by overexpression of *HRD* in *Arabidopsis*. (*A*) Drought-resistance tests of *Arabidopsis* WT and the *hrd-D* mutant line, treated for 9–12 days without water. The first row is at 9 days of dehydration (DOD), followed by plants treated for 11 and 12 DOD that were subsequently watered to reveal surviving plants. (*B*) Mutant *hrd-D* and WT *Arabidopsis* treated at 300 mM NaCl concentrations, showing bleached/dead plants and surviving *hrd-D* plants.

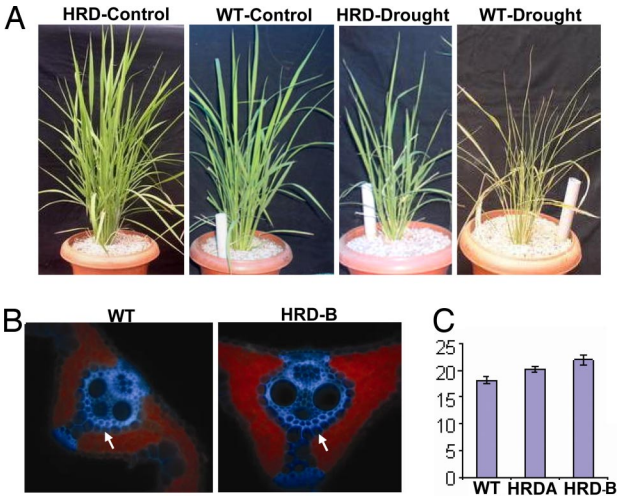

Fig. 4. Phenotype of *HRD* overexpression in rice. (*A*) Rice *HRD* overexpression line compared with WT Nipponbare under well watered (control) and water-stress (70% field capacity) conditions. (*B*) Leaf cross-section of WT and *HRD* overexpression lines, observed under fluorescence microscope, revealing red chlorophyll fluorescence and blue vascular bundles surrounded by the bundle sheath cells marked with an arrow. (*C*) Number of bundle sheath cells in WT compared with *HRD* overexpressors, which show significant increase ($n > 5$, $P = 7.5 \times 10^{-10}$).

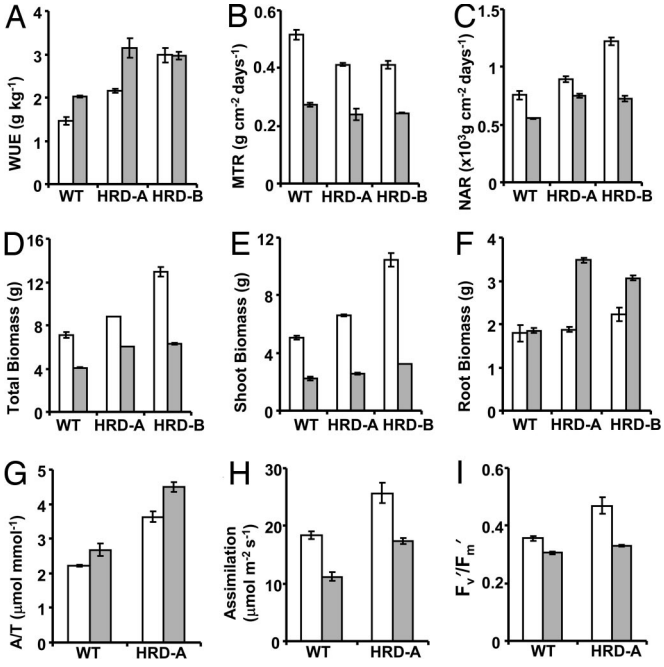

Fig. 5. Physiological analyses of rice *HRD* overexpression lines showing improved WUE. (*A* and *B*) The *HRD* lines and WT Nipponbare tested under well watered (white) and drought stress (shaded) conditions. Bars indicate SE ($n > 3$). All parameters are significant at 1% with calculated *P* values shown for *HRD* vs. WT. (*A*) WUE by gravimetric determination ($P = 1.6 \times 10^{-04}$). (*B*) MTR ($P = 2 \times 10^{-2}$). (*C*) NAR ($P = 2.27 \times 10^{-5}$). (*D*) Total biomass ($P = 9.9 \times 10^{-10}$). (*E*) Shoot biomass ($P = 7.4 \times 10^{-6}$). (*F*) Root biomass ($P = 1 \times 10^{-7}$). (*G*) Instantaneous WUE ($P = 1.4 \times 10^{-4}$). (*H*) Carbon assimilation ($P = 2.9 \times 10^{-4}$). (*I*) Relative quantum yield of PSII at steady-state photosynthesis ($P = 2 \times 10^{-3}$).

drought-resistance assay showed significant drought resistance, even when mild dexamethasone treatments were used (only treated from bottom by watering) that did not induce the typical *hrd-D* small dark green plant phenotype (SI Fig. 9 and SI Table 3). These results supported that the stress resistance was not a secondary result of the smaller-structured *hrd-D*-like mutant plants but a resistance mechanism induced by the *HRD* gene.

To get further insight on the mechanism of *HRD* action, the expression of *HRD* was studied revealing promoter expression in inflorescence tissue, including petals, young inflorescence stem, mature pollen, and seed (SI Fig. 10). Microarray analysis of the *hrd-D* mutant compared with WT (SI Fig. 11 and Gene Expression Omnibus accession no. GSE8936) revealed differentially expressed genes that were compared with publicly available microarray data.

HRD Overexpression in Rice Improves Drought Resistance and WUE. To validate and dissect the physiological mechanisms of drought stress resistance attributable to *HRD* expression in a crop plant, an overexpression construct with the *HRD* gene under control of the CaMV35S promoter (SI Fig. 6*B*), was transformed into rice (*Oryza sativa*, subspecies *japonica*) cultivar Nipponbare. The transformants were checked for expression of the gene (data not shown) and the selfed progeny of two independent lines (referred to as rice *HRD* lines) used for phenotypic and stress physiological assays. The transformants did not show any reduction in growth, seed yield, or germination when grown under normal greenhouse conditions, but they surprisingly revealed a significant visual increase in leaf canopy with more tillers (Fig. 4*A* and SI Fig. 12*D*). The rice *HRD* lines displayed a deeper green color as in *Arabidopsis* and were studied in more detail. In thin sections of leaf tissue examined under a fluorescent microscope, no significant number of mesophyll cells or fluorescence was observed. However, the *HRD* lines showed a significant consistent increase in number of bundle sheath cells (Fig. 4*B*), measured across the veins at equivalent stages and positions in the plant genotypes.

In replicated pot experiments, a control set of well watered/irrigated WT and *HRD* lines (T3 generation lines) were maintained at 100% field capacity (the maximum amount of water that the soil can hold), and another set of drought-stressed plants was kept at 70% field capacity. The *HRD* lines displayed distinctive drought resistance, whereas the WT plants showed typical stressed symptoms of leaf rolling and drying (Fig. 4*A*).

In gravimetric estimations of WUE (14), the rice *HRD* lines revealed a significant 50–100% increase under well watered control conditions and an ≈50% increase under drought stress (Fig. 5*A*). These lines also showed a reduction in mean transpiration rate (MTR) that was more pronounced under well watered conditions (Fig. 5*B*). These data were substantiated by lower stomatal conductance in *HRD* lines (SI Fig. 12*B*) shown by infrared gas analyzer measurements (15). The net carbon assimilation rate (NAR) of these lines was significantly higher than WT under both nonstress and stress conditions (Fig. 5*C*). The lower MTR and high NAR in *HRD* lines suggest that better mesophyll efficiency contributes to the higher biomass accumulation (Fig. 5*D*).

Photosynthetic gas exchange parameters (15) showed that *HRD* lines maintained a significantly higher rate of photosynthetic carbon assimilation compared with WT under both drought stress (55%) and well irrigated conditions (40%) (Fig. 5*H*). Higher rates of photosynthesis associated with lower levels of transpiration (SI Fig. 12*A*) resulted in a significant 65% increase in instantaneous WUE (Fig. 5*G*), consistent with the gravimetric WUE estimates (Fig. 5*A*). The better photosynthetic capacity of the rice *HRD* lines is associated with higher efficiency of the photosystem II (PSII) reaction center in the light as represented by F'_v/F'_m (16) (Fig. 5*I*). However, because there is no significant difference between plants in maximum quantum yield of PSII (F_v/F_m), with an average value of 0.804 ± 0.0036, the drought treatment did not damage the PSII reaction center

(17). This affirms that the higher PSII reaction center efficiency of the *HRD* lines contributes to the increased photosynthetic capacity.

The total biomass (root and shoot) of rice *HRD* lines, measured gravimetrically, shows a very significant increase of ≈50% under drought stress and 25–80% under nonstress control conditions (Fig. 5*D*). Increased shoot (leaf and stem) growth is the major contributor for the higher biomass under nonstress conditions (Fig. 5*E*), whereas under drought, increased root growth (>60%) (Fig. 5*F*) contributes to the total biomass.

Discussion

The *HRD* gene belongs to a class of AP2/ERF-like transcription factors, classified as group IIIb in a recent comprehensive classification of the AP2/ERF family (18). The related *DREB*/*CBF* genes that provide abiotic stress tolerance on overexpression (19, 20) belong to the group IIIc, but no phenotypic function has yet been attributed to the four *Arabidopsis* and six rice group IIIb members. Our analysis of *HRD* promoter expression (SI Fig. 10) supports the publicly available expression data in Genevestigator (21), showing that the *HRD* gene is expressed in inflorescence tissue including petals, inflorescence stem, mature seed, and pollen. Therefore, the gene is probably involved in the maturation of inflorescence stage processes that require protection of tissue against desiccation. Likewise, overexpression of the *HRD* gene in *Arabidopsis* confers drought resistance and salt tolerance, two abiotic stress components of desiccation tolerance (22).

Microarray analysis of *HRD* overexpression revealed significant differentially regulated clusters of genes represented as Gene Ontology (GO) terms that correlated with corresponding drought-regulated "GO clusters" (SI Fig. 11). *HRD* overexpression results in the induction of GO clusters normally expressed under drought stress, such as response to water deprivation and osmotic stress, supporting the induction of a drought adaptive mechanism by *HRD*. In addition, GO clusters repressed under drought are up-regulated by *HRD*, suggesting a protective influence on essential processes, such as protein biosynthesis and carbohydrate metabolism. Overexpression of the *DREB*/*CBF* group IIIc genes of the AP2/ERF family (23) shows similarity to *HRD* in regulation of stress response genes. However, *HRD* displays differences in the differential expression of some GO clusters, such as protein biosynthesis and other specific genes, which probably contribute to its different unique functions in *Arabidopsis* and rice.

Genes for abiotic stress tolerance have often been identified by ectopic expression in vegetative tissue, which can be easily tested in drought/salinity assays. In this way, the *Arabidopsis DREB1A* gene, normally induced by cold/salt stress, also confers drought stress resistance upon overexpression (20). Under natural situations in plants, the expression of stress resistance genes is controlled to respond adaptively to stress, or the stress resistance genes are expressed at specific developmental stages and in tissues providing stress resistance, but by ectopic expression they can exhibit the phenotype in another target tissue or condition. Thus, the *Arabidopsis HRD* gene, normally active in inflorescence-stage tissue, by ectopic expression exhibits a vegetative-stage phenotype of enhancement in root and leaf structure. The role of root and leaf structure has been recognized as an adaptive mechanism for drought resistance and WUE in crops such as rice (9).

The overexpression of *HRD* in rice generates plants with significantly higher biomass, independent of drought stress. With the increase in shoot biomass in the *HRD* lines, there is a reduction in the specific leaf area, the leaf area per unit dry weight (SI Fig. 12*C*), suggesting an increase in leaf thickness or tissue density. A supporting observation is the increased number of cells in the bundle sheath (Fig. 4*B*) that might also contribute to increased photosynthetic assimilation. In addition, there is also an increase in total leaf area duration (LAD) (SI Fig. 12*D*), suggesting that the increase in shoot biomass through leaf thickness/density and area is probably attributable to enhanced leaf mesophyll growth, a characteristic of *HRD* overexpression in *Arabidopsis*. *HRD* overexpression increases root biomass under drought stress, indicating an ability to adapt by inducing roots to harvest the scarce water. The increases in photosynthesizing area and carbon assimilation would contribute significantly to canopy photosynthesis, resulting in high biomass. This result is probably related to an increase in leaf mesophyll, bundle sheath, and root cortical cells, enhancing the capacity of both source and sink tissue.

The *HRD* lines show consistent significant differences compared with the WT under well watered and drought conditions (Fig. 3). However, the two *HRD* lines exhibit slight differences, HRD-B with higher shoot biomass and LAD determining a higher WUE under well watered conditions, whereas HRD-A responds better to drought with increased root biomass and WUE, which may be related to the observed difference in transgene expression levels (data not shown). This indicates the range of phenotypes and mechanisms conferred by *HRD* with potential to engineer an adaptive response to environmental stress in the different tissues and growth stages required to provide plant protection.

Under drought stress, crop yield can be determined by WUE (24), the ratio between biomass produced and water transpired. At a given environmental condition, the total transpiration depends on the stomatal conductance, cuticular properties, and total leaf area, whereas biomass is determined by the canopy carbon gain, which is regulated by NAR and functional leaf area. Therefore, improvement in WUE could arise from changes in these components. Our experiments in rice show that *HRD* expression causes significant increases of instantaneous and whole-plant WUE in well watered and drought conditions, with a very remarkable increase of 100% in absence of drought and a consistent 50% increase under drought stress. However, water deficit has been shown to increase WUE in different crops (25), the extent of which depends on the efficiency of carbon gain under stress conditions.

Improvement of WUE in crops can be achieved by a decrease in stomatal conductance or increase in photosynthetic capacity (26). In *HRD* lines, lower stomatal conductance results in reduced transpiration rate, contributing to higher WUE. The lower stomatal conductance contributes significantly to low MTR at the whole-plant level (Fig. 5*B*), which indicates that the *HRD* gene plays a role in stomatal behavior, similar to *ERECTA*, a gene known to lower the stomatal conductance in *Arabidopsis* (7).

More noteworthy, *HRD* overexpression shows significantly higher NAR (Fig. 5*C*) and net photosynthetic carbon assimilation (Fig. 5*H*), exemplified under drought stress conditions. The major contributing factor for higher biomass and WUE, therefore, seems to be a higher NAR because of better mesophyll efficiency in the *HRD* lines. Higher net photosynthesis and relative quantum yield of PSII at steady-state photosynthesis (Fig. 5 *H* and *I*) reflect the higher mesophyll capacity in *HRD* lines. Although drought stress reduces the efficiency of the PSII reaction center similarly to other studies in rice (27), *HRD* overexpression maintains higher efficiency of open PSII reactions compared with WT, under both well irrigated and drought conditions, directly indicating the higher photosynthetic efficiency. The amount and activity of photosynthetic machinery per unit leaf area contribute to mesophyll efficiency and influence transpiration efficiency of biomass production (6). Higher efficiency could be related to the increased photosynthetic mesophyll cells in thicker leaves, as exhibited by overexpression of *HRD* in *Arabidopsis*, with a reduction of specific leaf area in rice *HRD* lines as observed before in another crop (28). Future analyses would show whether the observed *HRD*-regulated

categories of genes contribute to the enhanced drought stress resistance and WUE in rice.

WUE is a trait of importance to all crops in a water-limiting environment. The demonstration of engineering higher WUE and drought resistance in the monocot crop plant rice by expression of the dicot *HRD* gene supports broad applicability of the gene and underlying mechanism of enhanced photosynthetic assimilation in a wide range of crop plants under well irrigated and drought-stressed environments. This would be able to contribute to increased biomass for grasses and other crops useful for carbon fixation and use as a sustainable bioenergy source (5). Improved WUE and drought resistance in grain/seed crops would also contribute to maintaining crop yields with limited water for sustainable agricultural production.

Materials and Methods

***Arabidopsis* Plant Growth and Phenotyping.** The *Arabidopsis* plants used are in ecotype Wassilewskija (Ws-3) and were grown in the greenhouse at ≈22°C. For growth measurements, fresh weight of plants was determined immediately after harvesting, and samples were oven-dried at 65°C for 1 week to obtain dry weight. To measure the strength of the *hrd-D* and WT roots, we used 5-week-old plants grown in well watered soil in Aracon containers (BetaTech, Gent, Belgium). For each plant, a nylon cord was tied to the base of the stem, and, using an attached pulley with a container, sand was gradually added until the plant roots were pulled out or broke from the anchored pot below. The weight of sand required to pull the plants out was recorded, and averages were calculated for the different genotypes.

For leaf structure analysis, cryo-fracture scanning electron microscopy was done as described in ref. 12. Cross-sections of the *Arabidopsis* roots from 6-week-old plants were made by free-hand sections with a razor blade, stained with phloroglucinol·HCl (1% wt/vol phloroglucinol in 6 M HCl), and observed under dark field microscope (29).

DNA Analysis and Plant Transformation. Plant DNA was isolated, 10 ng of which was used for TAIL PCR (10), and the flanking DNA was sequenced to reveal the insert position in the *Arabidopsis* genome by using BlastN (30). Fragments encoding the *HRD* gene (At2g36450) were amplified from *Arabidopsis* ecotype Columbia genomic DNA by using *pfu* DNA polymerase with primers HRDf (5′-CGGATCCATGCAAGGAACCTCCAAAGAC-3′) and HRDr (5′-CGTCGACGGTTTGTTTAACTATCATGG-3′), cloned into the pGEM-T Easy vector (Promega, Madison, WI), and sequenced before digestion and ligation to the binary vectors.

For *Arabidopsis* transformation, the 35S:HRD gene construct was made (SI Fig. 6). The oligonucleotides introduced BamHI and SalI restriction sites to the *HRD* fragment at the 5′ and 3′, respectively, which were used to ligate the 555-bp coding region fragment to compatible sites in the pNEW1 binary vector (N.M.-M. and A.P., unpublished data), in between a CaMV35S promoter (31) and the nopaline synthase terminator (tNOS). To make an overexpression construct for rice transformation (SI Fig. 6), the binary vector pMOG22 (Zeneca-Mogen, Leiden, The Netherlands) was used, containing a chimeric CaMV 35S-hygromycin phosphotransferase-tNOS for hygromycin selection during plant transformation. To generate the steroid-inducible 35S::HRD-GR constructs (SI Fig. 6), an *HRD* gene fragment was amplified (using *pfu* DNA polymerase; Promega) from *Arabidopsis* ecotype Columbia DNA by using the oligonucleotides RP6 (5′-TTATTTCTAGAATGCAAGGAACCTCCAAAGAC-3′) and RP7 (5′-TTATTAGATCTTGGAAAATTCCACAAGTAATCG-3′) that introduced XbaI and BglII restriction sites at the 5′ and 3′, respectively. The *HRD* gene and other fragments, including a 0.85-kb BglII–XhoI GR fragment obtained from pMTL23 (11), were used in multipoint ligations with compatible ends to assemble the construct.

The constructs were introduced into *Arabidopsis* by using the floral dip transformation method (32); the seeds were selected for transformants on medium with 50 mg/liter kanamycin or 20 mg/liter hygromycin and subsequently transferred to the greenhouse. *Agrobacterium*-mediated transformation of *O. sativa* ssp. *japonica* cv. Nipponbare, using the AGL-1 *Agrobacterium* strain, plant regeneration, and growth were performed as reported in ref. 33. Regenerated transgenic plantlets were transferred to the greenhouse and grown in hydroponic culture with a regime of 12 h of light, 28°C, 85% relative humidity and 12 h of dark, 25°C, 60% humidity.

Gene Expression Analyses. Total RNA for RT-PCR was isolated from mature green rosette leaves derived from 4-week-old *hrd-D* mutant and WT plants by using the TRIzol reagent as described by the manufacturer (Invitrogen, Carlsbad, CA). Approximately 1 μg of total RNA was used for DNase I treatment and cDNA synthesis (using SuperScript II reverse transcriptase; Invitrogen) as described by the supplier (Invitrogen). The cDNA was diluted 50 times and used for amplification by using specific oligonucleotides for the actin gene to equalize the concentrations of the cDNA samples (12). Subsequently, the diluted cDNA was used to perform a PCR by using oligonucleotides HRDf and HRDr to amplify the At2g36450 gene. The reaction conditions for PCR included a denaturing step of 95°C for 3 min followed by 35 cycles of 1 min at 95°C, 1 min at 55°C, and 1.5 min at 72°C, ending with an elongation step of 5 min at 72°C. For the control PCR with actin oligonucleotides, 30 amplification cycles were used.

***Arabidopsis* Abiotic Stress Experiments.** For the drought resistance experiments, the *hrd-D* mutant was compared with WT ecotype Ws as described in ref. 12. The experiments were repeated three times. To screen for salinity tolerance (13), we used seedlings grown at a density of one to two plants per 2-cm pot in a tray with potting soil (Hortimea, Elst, The Netherlands). Nutrients (Hydroagri, Rotterdam, The Netherlands; electrical conductivity 2.6) were supplied 2 weeks after germination, and 3 weeks after germination, the plants were treated with NaCl (200, 250, and 300 mM) at intervals of 3 days for three applications and were monitored for bleaching during the next 2 weeks. Survival rates were counted on day 10 after the third application of NaCl. The experiment was repeated three times.

Rice Physiological Analyses. To test for physiological parameters of rice lines under water-limited conditions, gravimetric analysis was done to measure WUE (14). Rice seedlings of T_3 generation lines, grown in small peat pots for 2 weeks in the greenhouse at 27°C and 60–65% relative humidity, were transplanted into 5-liter pots with soil:sand:peat (1:1:1) of known weight, and the soil surface was covered with a small stone mulch to restrict direct evaporation from the soil surface. A poly(vinyl chloride) tube with holes was inserted in the pots to ensure that water reached the root zone. A set of plants was maintained in soil at 100% field capacity (FC), whereas another set was brought down to 70% FC gradually to impose drought stress. During the experimental period, the pots were weighed daily (0.1-g accuracy), and the difference in weight on subsequent days was corrected by adding water to maintain the required FC. In addition, filled pots without plants were monitored to calculate the water lost by evaporation due to the mulch. The water added during the experimental period of 55–75 days after sowing (DAS) was summed up to arrive at the cumulative water transpired (CWT). At the start of experiment (55 DAS), the pots were sealed to prevent run-off. Representative plants from both WT and *HRD* overexpressors ($n > 3$) were sampled to measure the initial biomass (B_{55}) (leaves, root, and stem were separately

oven-dried for 1 week at 70°C and weighed separately) and leaf area (LA_{55}) by using a leaf area meter (ΔT Devices, Burwell, England). Similar measurements B_{75} and LA_{75} were done at the end of the experiment (75 DAS).

We computed WUE (g kg^{-1}) = $(B_{75} - B_{55})$/CWT, assuming linear growth during the experimental period, where B_{75} and B_{55} are the total biomass (grams per pot) measured at 75 and 55 DAS, respectively. CWT is the cumulative water transpired (in grams) during the experimental period. LAD is the functional leaf area during the experimental period and calculated as LAD (cm^2 days) = $(LA_{55} + LA_{75})/2 \times (B_{75} - B_{55})$, where LA_{55} and LA_{75} are the leaf area (cm^2 per plant) measured at 55 and 75 DAS, respectively. MTR per plant was estimated from CWT (in grams) and total transpiring LAD (cm^2 days) during the experimental period; MTR (g cm^{-2} day^{-1}) = CWT/LAD. NAR per plant was estimated by taking total biomass and total photosynthesizing area accumulated during the experimental period: NAR (g cm^{-2} day^{-1}) = $(B_{75} - B_{55})$/LAD.

Gas exchange parameters were measured on the fully expanded leaf second from the top of the WT and HRD-A lines by using a portable infrared gas analyzer (LI-6400; LI-COR, Lincoln, NE) (15). The parameters net photosynthetic rate (A) and transpiration rate (T) were used to calculate the instantaneous WUE (ratio A/T), which is the amount of CO_2 fixed per unit amount water lost by transpiration. The measurements were made at an ambient CO_2 concentration of 360 μmol mol^{-1} and photosynthetic photon flux density of 1,800 μmol m^{-2} s^{-1} by using LICOR light source and a chamber temperature of 28°C ± 0.5. Photosynthetic gas exchange and chlorophyll-a fluorescence were measured simultaneously (14). The maximum quantum yield of PSII (F_v/F_m) was measured in attached dark-adapted leaves. The relative quantum yield of PSII was calculated as $F'_v/F'_m = (F'_m - F'_o)/F'_m$, where F'_o is the minimal fluorescence of light adapted leaf, and F'_m is the maximal fluorescence during saturating light. All experiments were tested for significance of parameters by ANOVA, comparing the *HRD* lines vs. WT and control vs. drought treatments. Statistical analysis was done with Excel software (Version 7.0; Microsoft, Redmond, WA) and Gstat (www.gstat.org).

For leaf structure analysis, cross-sections of the third fully expanded leaf from the top of the main tiller from 6-week-old *HRD* overexpressors and WT plants were made by free-hand sections with a razor blade and then observed for chlorophyll autofluorescence under ×40 magnification by using a UV fluorescence microscope (Zeiss, Augsburg, Germany).

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