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Methods

Combining UAV-RGB high-throughput field phenotyping and genome-wide association study to reveal genetic variation of rice germplasms in dynamic response to drought stress

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Summary

- Accurate and high-throughput phenotyping of the dynamic response of a large rice population to drought stress in the field is a bottleneck for genetic dissection and breeding of drought resistance.
- Here, high-efficiency and high-frequent image acquisition by an unmanned aerial vehicle (UAV) was utilized to quantify the dynamic drought response of a rice population under field conditions. Deep convolutional neural networks (DCNNs) and canopy height models were applied to extract highly correlated phenotypic traits including UAV-based leaf-rolling score (LRS_uav), plant water content (PWC_uav) and a new composite trait, drought resistance index by UAV (DRI_uav).
- The DCNNs achieved high accuracy (correlation coefficient $R = 0.84$ for modeling set and $R = 0.86$ for test set) to replace manual leaf-rolling rating. PWC_uav values were precisely estimated (correlation coefficient $R = 0.88$) and DRI_uav was modeled to monitor the drought resistance of rice accessions dynamically and comprehensively. A total of 111 significantly associated loci were detected by genome-wide association study for the three dynamic traits, and 30.6% of them were not detected in previous mapping studies using nondynamic drought response traits.
- Unmanned aerial vehicle and deep learning are confirmed effective phenotyping techniques for more complete genetic dissection of rice dynamic responses to drought and exploration of valuable alleles for drought resistance improvement.

Introduction

Drought stress is one of the major factors limiting crop production (Basu *et al.*, 2016), and rice is one of the most drought-susceptible crops (Fischer *et al.*, 2012), consuming 50% of the freshwater resources in China (Luo, 2010). Grain yield reduction caused by drought stress ranged from 12% to 46% (Ouk *et al.*, 2006). Thus, there is an urgent need to breed drought-resistant and high-yielding rice adapted to future climate change (Trenberth *et al.*, 2014) to guarantee grain production (Tester & Langridge, 2010; Tardieu *et al.*, 2017).

Nowadays, the genomics revolution has improved plant breeding efficiency (Furber & Tester, 2011; Barabaschi *et al.*, 2016).

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However, high-throughput drought resistance phenotyping in the field remains a technical bottleneck, which has limited our ability to dissect the genetic basis of quantitative traits (Houle *et al.*, 2010; Araus & Cairns, 2014). Drought stress causes arrays of morphological and physiological changes, including leaf-rolling, decreased leaf water potential and increased stomatal resistance. Leaf-rolling is one of the most obvious responses to water deficit (Kadioglu & Terzi, 2007; Kadioglu *et al.*, 2012), and it is linearly related to leaf water potential (O'Toole & Cruz, 1980). Leaf-rolling protects the photosynthetic functionality of rice leaves from severe damage (Nar *et al.*, 2009), and maintains considerable yield under drought stress conditions (Basu *et al.*, 2016). Based on the leaf-rolling trait, some quantitative trait loci (QTLs) have been mapped for drought resistance (Price *et al.*, 1997), and a key gene modulating rice leaf-rolling was characterized (Zhang *et al.*, 2009). Leaf-rolling was commonly

assessed visually by multiple raters (O'Toole & Cruz, 1980), which means a heavy workload and poor timeliness in variable field conditions. Besides, inter- and intra-rater cognitive variability can result in strong subjectivity (Ghosal *et al.*, 2018). Rui *et al.* (2018) used a back-propagation (BP) neural network to identify leaf-rolling of the pot-grown rice plant in imaging chambers. Baret *et al.* (2018) investigated the relationship between manual leaf-rolling scores and changes in canopy structure using the upward-looking hemispherical photography RGB images taken from the bottom of the maize crop. However, these methods have not achieved automatic high-throughput leaf-rolling scoring in the field. It is essential to swiftly acquire phenotypic traits of a large germplasm population because drought responses and symptoms are strongly dependent on rapid changes in environmental conditions (Kadioglu *et al.*, 2012). Therefore, the current phenotyping approaches cannot meet the high-throughput requirements, especially for the quantification of dynamic drought responses.

Methods for automatic, objective, precise, and continuous quantification of drought-related traits such as leaf-rolling score are eagerly needed. Many studies have focused on image-based phenotypic traits (Singh *et al.*, 2020). Yang *et al.* (2014, 2020) developed a glasshouse high-throughput plant-wise phenotyping platform and leaf, yield traits scorers to automatically extract image traits. Duan *et al.* (2018) extracted a series of image features to describe rice morphology and color precisely, thereby discriminating drought-sensitive and drought-resistant rice. Furthermore, phenotypic traits were combined with genome-wide association studies (GWAS) to reveal the genetic architecture of drought resistance in rice (Guo *et al.*, 2018) and cotton (Li *et al.*, 2020a), to detect loci for rice root traits (Courtois *et al.*, 2013) and to dissect dynamic genetic architecture in rapeseed (Li *et al.*, 2020b). But these platforms and technologies developed for glasshouse or pot-grown plants can hardly be applied in the field owing to complicated imaging conditions such as illumination and soil background. In outdoor conditions, unmanned ground vehicles (UGVs) such as the LeasyScan at Telangana, India (Vadez *et al.*, 2015) moves scanners above the pot-grown crop canopy, which allows higher throughput than imaging chambers. Although there are also UGVs designed for automated field phenotyping, such as the LemnaTec Field Scanalyzer installed at Rothamsted Research of UK (Harpender, UK) (Virlet *et al.*, 2017), their scanning area is fixed and limited, resulting in high deployment expense and poor timeliness for a large germplasm population. Thus, field-based methods for fast, accurate and robust phenotyping are essential for genetic studies and breeding complex traits such as drought resistance.

Unmanned aerial vehicle (UAV) remote sensing provides flexible platforms that can be quickly deployed for field-based drought phenotyping (Yang *et al.*, 2017). A variety of imaging techniques can be integrated into the UAV by carrying lightweight sensors (Li *et al.*, 2014b), among which the consumer-grade camera (Zhang *et al.*, 2020a) is a cost-effective solution to capture high-resolution images. Thus, low-altitude UAV remote sensing of plant canopies permits nonintrusive and high-throughput plant phenotyping (Tattaris *et al.*, 2016). Besides, the rapidly developing tools of machine learning and deep learning can extract patterns and features from the large image datasets for drought stress identification and

quantification (Singh *et al.*, 2016; Pound *et al.*, 2017). Deep learning has been used in extracting image-based traits to identify and classify foliar stresses in soybean (Ghosal *et al.*, 2018) and to segment rice panicles (Xiong *et al.*, 2017). An open-source deep learning tool was introduced to conduct common plant phenotyping tasks such as leaf counting, segmentation and classification (Ubbens & Stavness, 2017). Combined with UAV images, deep learning was applied to segment purple rapeseed leaves (Zhang *et al.*, 2020b), detect and count citrus trees (Ampatzidis & Partel, 2019), and enable larger-scale aerial phenotyping and precision agriculture (Bauer *et al.*, 2019). Therefore, the combination of UAV remote sensing and deep learning provides a new opportunity for efficient drought response quantification.

The overall goal of this research was to develop an efficient phenotyping method with high spatial and temporal resolution UAV images to monitor, dynamically and nondestructively, morphological and physiological changes and to dissect genetic variation in a large rice population grown in a natural field subjected to drought stress conditions. The specific objectives were to build a deep convolutional neural network (DCNN) to assess leaf-rolling and its dynamic change; to calculate the relative plant water content (PWC_{uav_R}), and propose a new comprehensive trait, drought resistance index (DRI_{uav}), based on the UAV traits to dynamically monitor the water status of a rice population under drought stress; and to combine GWAS with dynamic UAV traits and haplotype analysis in order to find useful alleles for improvement in drought resistance.

Materials and Methods

Field experiment design and ground-truthing

The drought phenotyping experiment was conducted in the drought resistance testing station (30°28'15.84"N, 114°21'6.42"E) of Huazhong Agricultural University, Wuhan, China. To implement drought treatment, the rice field (size 55 m × 15 m) can be covered by a moveable rain-off shelter to maintain drought stress on rainy days. This study was based on a 2 yr trial at this experimental station. In 2018, the rice field was treated for 30 d drought stress conditions before the heading stage, and then irrigated for rice recovery. The drought treatment at time point D0 was achieved by stopping irrigation, draining water in the ditch, and closing the shelter if raining to make the field dry down slowly. The rice plants grew normally at the early stage (D1–D8) after the treatment, therefore the phenotypic data collected at this stage were used as the initial status of rice (control). During the mild drought stress (D10–D14), mild leaf-rolling happened in some sensitive rice accessions and the rice stopped to gain biomass. Then more rolling leaves and yellow leaves appeared gradually during the moderate drought stress (D15–D20). Finally, the leaves of most rice accessions rolled as a closed cylinder (O'Toole & Cruz, 1980) under severe drought stress (D21–D27). The soil water content was monitored by 60 soil moisture sensors evenly buried in the field to confirm the homogeneity of soil properties. A total of 240 accessions with two replications (480 plots) were phenotyped before drought

stress, during drought stress and after recovery. Information on these accessions is provided in Supporting Information Table S1. In 2019, 78 accessions with three replications (234 plots; see Table S2) were phenotyped as additional data. Each plot (size 1 m²) contained 20 rice plants evenly arranged in four rows and five columns. Plots were separated by one row of purple rice plants. The block design was completely randomized. The manual measurements for ground-truthing consisted of visual leaf-rolling scoring (illustrated in Fig. S1) and plant water content measurements (Notes S1; Table S3).

Ultra-high resolution UAV image collection and preprocessing

High-resolution RGB images were collected with a Sony RX1R II consumer-grade camera (Sony Electronics Inc., Tokyo, Japan) mounted on a DJI Matrice 600 Pro UAV (SZ DJI Technology Co., Shenzhen, China). The UAV imaging platform (Fig. 1a) and its configuration information can be found in Notes S2. Based on a UAV flight test with manually controlled height varying from 15 to 30 m above ground, the UAV flying height was finally set to 20 m above ground level with no disturbance to the rice leaves. The UAV observation covered the control, drought treatment and recovery stages. The images were captured at most three times (morning, midday and afternoon) each day at the time with obvious phenotypic differences (detailed image acquisition time is listed in Table S4).

Approximately 80 RGB images for each flight were used for the photogrammetric process with PHOTOSCAN software (Agisoft LLC, St Petersburg, Russia) to generate digital orthophoto maps (DOMs) and digital surface models (DSMs). The vignetting and geometric distortion were corrected (Zheng *et al.*, 2009; Kelcey & Lucieer, 2012) in the PHOTOSCAN software based on the camera sensor parameters before performing the photogrammetric processing. To obtain accurate geographical references (Gómez-Candón *et al.*, 2014), nine ground control points (GCPs) were measured by a global navigation satellite system real-time kinematic (GNSS RTK) receiver (Emlid Reach RS; Emlid Ltd, Saint Petersburg, Russia), and introduced into the reference panel of PHOTOSCAN software for GCP management (Notes S3) and georeferencing (James *et al.*, 2017). The GCPs at different heights were evenly distributed both in the field and on the ground (Fig. 1b) to control the vertical accuracy. The DOM and DSM (Fig. 1b) of each flight were exported in TIFF format. The ground sampling distance (GSD) of the DOM and DSM were 0.27 and 1.09 cm pixel⁻¹, respectively.

To build the dataset for DCNN modeling, the image patch of each rice plot needs to be cropped from the DOMs. Thus, a region of interest (ROI) shapefile was created in ARCMAP 10.3 (Esri Inc., Redlands, CA, USA) and 0.5 m × 0.5 m rectangles were drawn in the central area of each 1 m × 1 m rice plot (Fig. 1c). The rectangles were smaller than the plot size to reduce edge effects. The cropped patch sizes varied around 185 × 185 pixels as a result of minor differences in GSD. The dataset of image patches for each rice plot was built from the 12 DOMs (Table S4) simultaneously collected with the manual leaf-rolling scores

(LRS_m) (Fig. 1d,e). The other DOMs throughout the control, drought and recovery stages were also processed to generate time-series image patches for dynamic leaf-rolling scoring. The corresponding LRS_m values were used as the ground-truth label to annotate each image patch. In all, 2767 labeled patches (Table S5) were randomly split into training set and validation set in an 8 : 2 ratio before the data augmentation. Then a two-step data augmentation strategy (Notes S4) was adopted for both the training set and validation set.

Deep convolutional neural network for leaf-rolling scoring

A DCNN for automatic leaf-rolling scoring (DCNN-LRS) was built using the Keras library with TensorFlow (Abadi *et al.*, 2016) backend. All the modeling procedures were carried on a Linux platform with an NVIDIA Quadro GV100 GPU (32 GB) and a 4.00-GHz Intel Core i7-8086k CPU, with 64 GB of memory.

The network was built based on the VGG-16 (Simonyan & Zisserman, 2014) model and transfer learning (Kieffer *et al.*, 2017; Liu *et al.*, 2018) because the representations of the pre-trained VGG model generalizes well to other datasets through transfer learning (Simonyan & Zisserman, 2014; Wang *et al.*, 2017; Singh *et al.*, 2018), and pretrained networks are quite competitive against training from scratch (Kieffer *et al.*, 2017). In this study, the VGG-16 model pretrained on ImageNet (Krizhevsky *et al.*, 2017) without fully connected (FC) layers was used for transfer learning. The input size was changed to 160 × 160 × 3 to match the size of image patches. Then a global average pooling (GAP) layer was added behind the bottleneck layer (size of 5 × 5 × 512) to reduce the feature dimensions. Three FC layers with sigmoid activations were appended behind the GAP layer to do the feature fusion and regression. Unlike the original VGG network designed for multiclass classification with softmax activation in the last FC layer, the network architecture (Fig. 1f) in this study was designed to solve the numerical regression of leaf-rolling score. Therefore, sigmoid activation was used in the last FC regression layer (Lathuilière *et al.*, 2019), and the dropout regularization method was deployed after the first two FC layers to reduce overfitting (Srivastava *et al.*, 2014; Krizhevsky *et al.*, 2017). In detail, the dropout rate was set at 20%, and the sizes of the three FC layers were 512, 512 and 1, respectively. Finally, the output value of the last FC layer × 5 was used as the LRS_uav value as the manual score ranged from 1 to 5.

The DCNN-LRS was trained and validated with the dataset collected in 2018. There were 480 plots for 240 accessions with two replications, in which the 120 accessions were manually scored simultaneously with the image collection as a result of the low efficiency of manual measurement. In addition, the model performance was tested with the new datasets collected in 2019 (Table S6) to evaluate its generalizability. The pretrained VGG16 model without FC layers was used as a feature extractor. All the convolutional layers were frozen during the training procedure, and only the added FC layers were trained as fine-tuning does not seem to add any tangible improvement for VGG16 to justify additional training (Kieffer *et al.*, 2017). Mean squared

Fig. 1 Workflow of the unmanned aerial vehicle (UAV)-RGB high-throughput field phenotyping approach. (a) A DJI Matrice 600 Pro UAV platform (SZ DJI Technology Co., Shenzhen, China) carrying a consumer-grade RGB camera for the image collection of the rice field. (b) The digital orthophoto map (DOM) and canopy height model (CHM) of the rice field were generated for phenotypic trait extraction. (c) Dataset building by cropping image patches from the DOMs. (d) Image sets for rice germplasm resource 120 accessions were collected for the control, drought and recovery stages in 2018. (e) Image patch labeling with manual leaf-rolling scores (LRS_m). (f) Deep convolutional neural network (DCNN) architecture.

error (MSE) was employed as the loss function to be optimized for this regression model (Lathuilière *et al.*, 2020), and mean absolute error (MAE) was calculated as a performance metric. Adam optimizer (Kingma & Ba, 2014) was used with a 10^{-4} learning rate and 10^{-3} decay to adaptively optimize the training process. The batch size was set to 256, and each batch was generated by the data generator with real-time data augmentation. The model was trained for 300 epochs with 10 batches per epoch. A validation epoch was performed after every training epoch to calculate the loss and MAE for the validation set. The validation loss was used as the monitor metric, and the model file was only updated when validation loss decreased.

Biomass, plant water content and drought resistance index modeling

Drought stress decreases the fraction of stem, leaf and reproductive biomass (Eziz *et al.*, 2017) and loss of plant water content. Therefore, the above-ground biomass and plant water content traits can be applied to assess the drought stress response. The structure from motion (SfM) algorithm was adopted to build dense clouds and DSM of the rice field in the Agisoft PHOTOSCAN software. The canopy height model (CHM; Fig. 1b) generated from the DSM by subtracting the soil height was widely used to extract the above-ground biomass information (Han *et al.*, 2019). Here, a horizontal datum with a constant height value was used as the soil surface to be subtracted as the rice field elevation was stable. Then, above-ground volume (AGV_uav, m^3) was calculated by summing the CHM values within each plot. Empirical linear regression models were built from the AGV_uav to estimate the fresh weight, dry weight and plant water content (PWC_uav).

Based on these nondestructively measured dynamic traits, plant water content changes as a result of drought stress can be quantified. Here, the relative plant water content (PWC_uav_R, ratio of PWC_uav under stress : PWC_uav before stress) was calculated, in which the PWC_uav before stress was the initial phenotypic trait value just before the mild drought stress began. In this study, it was the eighth day after the drought treatment (D8). Under drought stress treatment, the PWC_uav_R decreases while the LRS_uav increases. Based on this pattern, a composite drought resistance index (DRI_uav) was proposed as the ratio of PWC_uav_R : LRS_uav, as shown in Eq. 1, to indicate the drought resistance based on the comprehensive drought responses:

$$\text{DRI}_{\text{uav}} = \frac{\text{PWC}_{\text{uav_R}}}{\text{LRS}_{\text{uav}}} \quad \text{Eqn 1}$$

Genome-wide association study

A GWAS was performed on a natural population consisting of 240 *Oryza sativa* accessions, including 135 indica accessions, 57 japonica accessions, 28 aus accessions, 14 admix accessions, and six VI accessions. Genotype data for the 240 accessions were reported in a previous study (Zhao *et al.*, 2015) and the data are available in the database RiceVarMap v.2.0 (<http://ricevarmap.ncpgr.cn/>).

The high-quality genotype data for 10 338 860 genomic variations (a minor allele frequency ≥ 0.05 and the number of accessions with minor alleles ≥ 6), covering the whole rice genome, were used to carry out GWAS using the linear mixed model (LMM), considering the kinship estimated by genetic similarities as a random effect provided by the FAST-LMM program (Lippert *et al.*, 2011). The kinship (K) matrix of all accessions was calculated using PLINK (Purcell *et al.*, 2007) based on 222 391 variations that were randomly selected from the genome (three variations per 5 Kb, a minor allele frequency > 0.01). A total of 1981 633 independent effective markers (N) were calculated using the GEC software tool (Li *et al.*, 2012). The genome-wide suggestive ($1/N$) and significant ($0.05/N$) thresholds of the GWAS (5.04×10^{-7} and 2.54×10^{-8}) were set to control the genome-wide type 1 error rate. Potential candidate genes within the 250 kb interval centered on the lead variation from the GWAS, and the variations ($P < 1 \times 10^{-7}$) were determined based on the local linkage disequilibrium (LD) level using the ‘--clump’ command of PLINK (Purcell *et al.*, 2007). Visualization of candidate gene associated mapping based on the GWAS results and pairwise LD analysis was performed by LDBLOCKSHOW (Dong *et al.*, 2020).

Results

Performance validation of DCNN-LRS

The performance of DCNN-LRS was evaluated during the training, validation and testing procedures. After 300 training epochs, the MSE loss was 0.579 for the training set and 0.646 for the validation set. The MAE decreased to 0.601 for the training set and 0.642 for the validation set, which is within one point of the five-point leaf-rolling scale. The DCNN-LRS learning curves are shown in Fig. S2. The trained DCNN-LRS was applied to predict the leaf-rolling score for the 120 accessions, and the accuracies of both replications were $R = 0.832$ (RMSE = 0.410) and $R = 0.870$ (RMSE = 0.311). Besides, bivariate correlation analysis was conducted between the two accession replications, the results were $R = 0.677$ for LRS_uav, and $R = 0.705$ for LRS_m, which confirmed the repeatability of the DCNN measurement. These results are listed in Table S7, and the DCNN-LRS model and the datasets are available online (Notes S5). The accurate LRS_uav trait can quantify the leaf-rolling severity with continuous measurement from 0 to 5 without dimension, and is thereby a more objective alternative to the manual measurements. Note that the LRS_uav value < 1 means that $< 20\%$ of the rice leaves in a plot become rolling, which can only be manually rated as 1. The data from continuous measurement may be more suitable for genetic analysis.

A robust model should be able to generalize to brand new datasets and still perform well. Therefore, the DCNN-LRS was tested with the additional 2019 dataset. The test dataset was collected at three drought stages. For each drought stage, five sets of images were collected to generate the DOMs, and the images were taken at 07:00 or 17:00 h, synchronized with the manual leaf-rolling rating. The example LRS results for the three drought

Fig. 2 Deep convolutional neural network (DCNN) for automatic leaf-rolling scoring (DCNN-LRS) test results for the new dataset collected in 2019. (a–c) Examples of the UAV-based leaf-rolling score (LRS_{uav}) results for the three drought stages, respectively, and the LRS_{uav} values are shown at the bottom of each rice plot. (d–f) Scatter plots and correlations of manual measurements and DCNN-LRS measurements for the three drought stages. The color of scatter points indicates their density distribution with decreasing density from red to blue.

stages are displayed in Fig. 2, in which the subtle variation of leaf-rolling severity was accurately quantified by the DCNN-LRS. The test result also confirmed the model's robustness and generalizability for further application.

LRS_uav trait reveals the dynamic leaf-rolling under drought conditions

The time-series image patches of six representative accessions (including two drought-sensitive, two intermediate and two drought-resistant accessions) during the drought stress stage are shown visualized in Fig. 3(a) with the LRS_uav displayed below. As drought stress became more severe, the differences in leaf-rolling severity among accessions were increasingly obvious. The sensitive accessions (such as MR185 and Lengshuigu in Fig. 3a) quickly reached a very serious leaf-rolling degree, while the LRS_uav of resistant accessions (such as C418 and Gang46B in Fig. 3a) increased slowly with a smaller range. The DCNN-LRS could quantify the leaf-rolling under different imaging conditions among multiple times of UAV flight. For instance, the test accuracy in 2019 was not influenced although the image patch brightness varied a lot (Fig. S3) as a result of the illumination difference, indicating the robustness and generalization ability of DCNN-LRS under unstable environmental conditions.

The trained DCNN-LRS was applied to automatically score leaf-rolling for each rice plot based on the time-series images collected in 2018. The traits of two replications were averaged to reduce random error. Heat maps (Fig. 3b) were drawn to visualize the dynamic LRS_uav traits of the 120 accessions. The leaf-rolling speed under drought stress conditions and the recovery speed after rewatering reflect the drought resistance ability at different stages. Overall, the LRS_uav became higher under continuous drought stress treatment, but lower in the recovery stage after rewatering. The leaves of the drought-sensitive accessions rolled rapidly under drought stress treatment, and hardly recovered after rewatering. By contrast, the leaves of the resistant accessions rolled slowly, and the LRS_uav values only increased with a smaller range under drought stress treatment and decreased to a normal value after recovery. Phenotypic differences in terms of LRS_uav among the accessions were very significant under moderate drought stress treatment, especially at the D17, D19 and D20 time points.

Plant water content and drought resistance index modeling results

Linear correlation analysis indicated that the AGV_uav trait, calculated from the CHM to predict the above-ground biomass, was strongly correlated with manually measured fresh weight, dry weight and plant water content (PWC_m) (Fig. 4a–c). The non-destructive PWC_uav trait modeled from the CHM throughout the control, drought and recovery stages revealed the dynamic change of the plant canopy water content. Moreover, the relative trait PWC_uav_R, calculated as the ratio of PWC_uav under drought stress to PWC_uav before stress, was sensitive to the water content changes caused by drought. First, the PWC_uav_R decreased as the drought stress got increasingly severe (Fig. 4d).

Then the PWC_uav_R increased to > 1 , suggesting that the rice plants were regained biomass and water content after rewatering. Similar phenotypic changes were found in the dynamic DRI_uav trait (Fig. 4e). Therefore, both the PWC_uav_R and DRI_uav revealed the dynamic changes of drought response, which cannot be measured by traditional destructive manual methods. Compared with PWC_uav_R, the composite trait DRI_uav showed more significant dynamic changes, and it may be more suitable for quantifying the comprehensive drought responses and assessing drought resistance.

Comparison of dynamic traits for resistant and sensitive accessions

We sought to better understand the characteristics and applicability of the indicators derived from UAV images (UAV traits) before, during and after drought stress. The dynamic phenotypic changes of the LRS_uav, PWC_uav_R and DRI_uav traits for the sensitive and resistant accessions were compared (Fig. 5). LRS_uav is an effective trait to discriminate the resistant and sensitive accessions (Fig. 5a). Before drought stress treatment, the LRS_uav value was 1.00 because the rice leaves of all accessions were not rolling under normal irrigation conditions. The LRS_uav gradually increased during the mild and moderate drought stress (D10–D20) and the difference between the sensitive and resistant accessions became significant. Then the LRS_uav reached its highest value in severe drought stress (D21–D27). After rewatering, LRS_uav differences were not significant before the Re10 time point. However, LRS_uav could discriminate the two groups from the Re15 time point, because the leaves of some sensitive accessions could not recover as quickly and completely as the resistant accessions from the serious drought damage, not even after rewatering. For the materials tested in this study, the LRS_uav of resistant accessions recovered better when the LRS_uav decreased to 1.50, which was nearly the healthy status before drought stress treatment, but the sensitive rice accessions could not recover well if LRS_uav was 2.00.

Dynamic PWC_uav traits are related to canopy leaf water status, and their changes under drought largely reflect stress responsiveness. We used the relative traits (ratios of trait values under drought stress to those before drought stress) to quantify the phenotypic changes. Most resistant accessions showed more reduced biomass percentages than sensitive accessions (Fig. 5b), which suggests a balance between growth and stress responses. When the DRI_uav value decreased to < 0.60 under drought stress treatment, significant differences in drought resistance appeared (Fig. 5c). When the DRI_uav value decreased to < 0.40 , rice plants suffered from severe drought stress. When the DRI_uav value increased to > 0.40 during the recovery stage, the rice leaves showed obvious recovery signs such as turgor regain and leaf expanding.

GWAS results support the effectiveness of UAV traits in genetic dissection of the dynamic drought response

To further evaluate the value of UAV traits in accurately quantifying the dynamic field drought response, we performed GWAS

Fig. 3 Visualization of the dynamic UAV-based leaf-rolling score (LRS_{uav}) change in the 2018 rice trial. (a) Image patches of two sensitive, two moderate and two resistant accessions in drought stress in which the LRS_{uav} values > 3 are dashed in a red border around the patches and the LRS_{uav} values of the six accessions are shown in the table below. (b) Heat map of LRS_{uav} for 120 accessions. The rows and columns stand for accessions and time points. D0, day of stopping irrigation; D1–D8, D10–D26 and Re3–Re16 refer to the control, drought stress and recovery stages of the 2018 trial, respectively. The phenotypic differences among the accessions were most significant during moderate drought stress, especially at the time points D17, D19 and D20.

Fig. 4 Empirical modeling of biomass fresh weight, dry weight and plant water content from the UAV-based above-ground volume (AGV_uav) of rice. The Pearson correlation coefficient (R), root mean square error (RMSE) and relative error (RE) are shown in the figure. (a) AGV_uav and manual fresh weight; (b) AGV_uav and manual dry weight; (c) AGV_uav and manual plant water content (PWC_m); (d) dynamic relative plant water content by UAV (PWC_uav_R); (e) dynamic drought resistance index by UAV (DRI_uav). Error bars represent means $\pm$ SD ($n = 120$).

for LRS_uav, PWC_uav_R and DRI_uav to examine whether these can efficiently detect drought resistance genetic loci (Table S8). A total of 31, 33 and 54 associated loci were detected by PWC_uav_R, LRS_uav and DRI_uav, respectively (Fig. 6a; Table S9). Among them, three loci were detected by both PWC_uav_R and LRS_uav, and four loci associated with LRS_uav were also detected by DRI_uav, but no common locus was detected by DRI_uav and PWC_uav_R (Fig. 6a). Approximately 30.6% of significant loci for the UAV traits were not detected in previous association or linkage mapping studies using nondynamic drought response traits. Of special note, 63% of the significant loci detected by UAV traits were not detected in a previous association study of image-derived drought tolerance traits using the same population but grown in glasshouse pots (Guo *et al.*, 2018) (Table S9), indicating the largely different genetic architecture of drought response under glasshouse and field conditions. More than 55% of significant loci identified in this study are overlapped with QTLs in previous studies, especially in field experiments (Table S10), suggesting that UAV traits are reliable in genetic analysis. For example, Locus 43 and Locus 25 were significantly associated with DRI_uav at multiple time points (DRI_uav_D17_PM, DRI_uav_D19_PM, DRI_uav_D20_PM) and LRS_uav at one time point (LRS_uav_D27_AM), respectively, and these two loci overlapped with two reported QTLs controlling leaf drying score (Likelihood of odds = 2.4) and leaf

rolling score (Likelihood of odds = 3.4), respectively, under field drought stress conditions (Yue *et al.*, 2005). Locus 70, significantly associated with DRI_uav_D20_PM, was colocalized with a reported QTL (*qDTY3.2*, Likelihood of odds = 9.9) controlling grain yield under field drought stress (Mishra *et al.*, 2013). These results strongly suggest that UAV traits can be used for genetic dissection of field drought resistance.

At the mild (D10–D14), moderate (D15–D20) and severe (D21–D27) drought stress stages, a total of 19, 56 and 30 significantly unique loci were detected by the UAV traits, respectively. Very few common loci were detected at any two of the three stages (Table S9), indicating that the genetic basis of drought response is significantly different at different drought stress stages. Compared with DRI_uav, more loci were detected by LRS_uav and PWC_uav_R at the mild drought stress stage. At the moderate drought stress stage, the number of loci associated with DRI_uav greatly increased, especially for the late afternoon time points, at which only a few loci were detected by LRS_uav and PWC_uav_R. However, very few loci were detected by the UAV traits at the severe drought stress stage, which is reasonable as most accessions showed completely irreversible leaf-rolling with a nonsignificant difference in water content at this stage. Moreover, more loci were detected in the late afternoon than at midday at all three stages (Fig. 6b). The GWAS results of the three UAV traits at time point D17_PM exemplified their power

Fig. 5 Dynamic changes of three traits for drought-sensitive and resistant rice accessions. (a) Leaf-rolling score by UAV (LRS_uav); (b) relative plant water content by UAV (PWC_uav_R); (c) drought resistance index by UAV (DRI_uav). D0, day of stopping irrigation; D1–D8, D10–D26 and Re3–Re16 refer to the control, drought and recovery stages of the 2018 trial. Error bars represent means $\pm$ SD ($n = 30$). Statistical significance was determined by Welch's t -test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

difference in detecting genetic loci (Fig. 6c–e). At this time point, Locus 38 was significantly (the most significant variation with $P_{\text{imm}} = 4.8 \times 10^{-12}$) associated with DRI_uav_D17_PM, Locus 109 was significantly associated with LRS_uav, and all significant variations were distributed in the intergenic region of two retro-transposon genes, whereas no significant associated loci were detected by PWC_uav_R. These results together suggest that the three UAV traits have advantages in dissecting the genetic basis of dynamic responses at different drought stress stages.

Usefulness of UAV traits in exploring natural variation of drought resistance genes

We further evaluated the usefulness of the UAV traits in detecting natural variations of known drought resistance genes that may have breeding potential. Compared with LRS_uav and PWC_uav_R, DRI_uav detected not only more genetic loci (Fig. 6a) but also more drought resistance-related genes located in the association loci. For example, several reported drought resistance related genes are located in DRI_uav-associated loci, including

OsSRO1c (You *et al.*, 2013) and *OsITPK2* (Du *et al.*, 2011) in Locus 42, *OsZIP12* (Joo *et al.*, 2014), and *OsEREBP2* (Serra *et al.*, 2013) in Locus 52, and *OsRCI2-5* (Li *et al.*, 2014a) in Locus 57 (Table S9). *OsSRO1c* is a direct target gene of the stress-responsive NAC transcription factor SNAC1 (Hu *et al.*, 2006), and confers drought tolerance by promoting stomatal closure and reducing water loss (You *et al.*, 2013). *OsSRO1c* was significantly associated with DRI_uav_D17_PM (Fig. 7a). One significantly ($P_{\text{imm}} < 7 \times 10^{-8}$) associated InDel variation in the *OsSRO1c* promoter showed tight linkage with the lead SNP ($r^2 > 0.8$). The 240 rice accessions were divided into two haplotype groups based on the significant InDel (frequency > 0.05 ; Fig. 7b). The Hap2 group (30 accessions) had a significantly higher DRI_uav_D17_PM than the Hap1 group (179 accessions). Moreover, nine Hap1 and 11 Hap2 accessions were randomly selected to examine *OsSRO1c* expression under normal and drought stress conditions at the reproductive stage. *OsSRO1c* expression was induced by drought stress, and the Hap2 accessions exhibited significantly higher relative expression levels and DRI_uav_D17_PM than Hap1 accessions (Fig. 7c; Table S11).

Fig. 6 Genome-wide association studies (GWAS) results for leaf-rolling score by UAV (LRS_uav), relative plant water content by UAV (PWC_uav_R) and drought resistance index by UAV (DRI_uav) of rice at time point D17_PM. (a) Venn diagram showing the numbers of loci associated with DRI_uav, LRS_uav, and PWC_uav_R, respectively. (b) The numbers of loci associated with DRI_uav, LRS_uav and PWC_uav_R at each time point are shown by a stacking bar chart. (c–e) Manhattan plot (left) and quantile–quantile plot (right) of the GWAS under a linear mixed model (LMM) for LRS_uav_D17_PM, PWC_uav_R_D17_PM, and DRI_uav_D17_PM, respectively.

Fig. 7 Natural variation of *OsSRO1c* was significantly associated with drought resistance index at time point D17_PM (DRI_uav_D17_PM) at the reproductive stage of rice under field conditions. (a) *OsSRO1c* Manhattan plot and pairwise linkage disequilibrium (LD) analysis (gene body and 2.5 kb upstream of the gene). The most significant variation in the gene is shown with a red diamond, and other variations are colored to display their LD with the most significant variation. The red dashed line indicates the suggestive threshold. (b) Haplotype groups of *OsSRO1c* in the association population based on the significant variation. DRI_uav_D17_PM distribution of each haplotype group is shown by boxplot. n denotes the number of accessions in each haplotype group. (c) Comparison of DRI_uav and relative expression level_R (drought and normal ratio) between two haplotype groups of *OsSRO1c*. In the boxplots (b, c), the black line within the box is the median, and the top and bottom of the box represent the 0.75 and 0.25 quartiles, respectively. The whiskers extend to data no more than $1.5 \times$ interquartile range, and data are indicated by dots. Statistical significance was determined by Welch's t -test: ***, $P < 0.001$; ****, $P < 0.0001$.

These data suggest that the InDel variation in the *OsSRO1c* promoter may cause differences in the drought-induced *OsSRO1c* expression level and contribute to drought resistance. In addition, *OsZIP12* (Joo *et al.*, 2014), *OsEREBP2* (Serra *et al.*, 2013) and *OsRCI2-5* (Li *et al.*, 2014a) were significantly associated with DRI_uav_D20_PM (see Fig. S4 and Notes S6 for details). Collectively, these results further supported that the UAV traits, especially the composite trait DRI_uav, are valuable in exploring natural variations of drought resistance loci or genes.

Discussion

Effectiveness and limitations of UAV traits in evaluating the dynamic drought response

In this study, we first proposed two UAV image-derived traits, including the LRS_uav and PWC_uav, to quantify the plant status during drought stress. These traits have their limitations in reflecting rice drought resistance. Our results indicate that proper traits should be chosen to quantify the drought response and resistance at different drought stress stages efficiently.

For leaf-rolling scoring, it took three raters at least 1.5 h to manually measure the LRS_m of 240 plots. The UAV flight of the rice field took less than 5 min, which was fast enough to capture accurate traits by avoiding the fluctuations of environmental

factors such as cloud or wind. The DCNN-LRS model was confirmed to be accurate, robust and objective to quantify leaf-rolling and replace visual scoring. More importantly, the high efficiency of UAV phenotyping makes dynamic monitoring with high temporal resolution possible. The LRS_uav trait was most significant to reflect the natural variation of rice drought resistance among accessions during drought stress treatment (Fig. 5a). However, there are some limitations for the LRS_uav trait. First, the leaf-rolling symptom at the early drought stage may not adequately reflect the overall drought resistance, because moderate leaf-rolling is a common drought avoidance mechanism under moderate drought stress conditions. This is supported by the fact that differences between sensitive and resistant accessions were not significant at early drought stages. Secondly, leaf-rolling is an unstable trait that is easily affected by temperature, humidity and light conditions in the field. Additionally, the LRS_uav value varies at different time points during the day. During the moderate drought stress period, the leaf-rolling phenotype is weakest early in the morning before sunrise. Then, the phenotype becomes more and more obvious as evaporation increases during the day, and the LRS_uav value peaks at midday or during the late afternoon (depending on the weather) during one day–night period. Therefore, the LRS_uav trait may not always accurately reflect drought resistance under complex field conditions. Besides leaf water content (or water potential), many other factors also

affect leaf-rolling and drought resistance. For example, smaller and thicker leaves may help plants avoid severe damage from drought stress (Fang & Xiong, 2015).

At the recovery stage, however, almost all resistant and sensitive rice accessions showed quick accumulation of water and biomass, and the differences among accessions were not significant. Therefore, the PWC_uav_R trait was not useful in evaluating drought recovery capability at the recovery stage. In addition, the biomass and water content changes were less obvious than the leaf-rolling symptom under drought treatment, so these two traits may only be used as referential indicators when LRS_uav is used as a major trait to quantify drought resistance.

We proposed the composite trait DRI_uav to comprehensively indicate the drought responses and reflect drought resistance. Under normal growth conditions, the DRI_uav value was mainly affected by the plant water content because the rice leaves were hardly rolling (Fig. 5c). Under mild and moderate drought stress, the DRI_uav value was strongly affected by the LRS_uav value because of the different capabilities in maintaining water potential among the rice accessions. Under severe drought stress, almost all the LRS_uav values were saturated to around 5, and the DRI_uav reached its lowest value. At the recovery stage, DRI_uav was influenced by the recovery status of both water content and leaf-rolling. Therefore, the DRI_uav is a comprehensive indicator reflecting drought resistance through the growing status under normal conditions and the dynamic responses during the drought stress and recovery process.

GWAS of dynamic UAV traits for rice drought resistance in the field

Drought resistance is a complex quantitative trait, which is largely influenced by the unstable environment. It is important to trace and accurately quantify the dynamic changes of drought-related traits under variable field conditions, so that stable association loci can be identified for drought resistance improvement. In this study, drought-related traits were frequently and dynamically phenotyped and subjected to GWAS. The difference in drought resistance among the rice population was successfully distinguished using the LRS_uav and DRI_uav traits (Fig. 5). Many genetic loci were detected at the moderate-severe and the severe stress stages by DRI_uav and LRS_uav, respectively (Fig. 6; Table S9). Meanwhile, more unique and common loci were detected by UAV traits at the moderate drought stress stage than at the mild or severe stress stage. Therefore, the moderate drought stress may be a key stage for genetic dissection and breeding of drought resistance in the field. Only one locus (Locus 29) associated with DRI_uav was identified at the mild and moderate drought stages, two associated loci were detected at the mild and severe drought stages, and a few loci (Locus 44, Locus 49, Locus 50) were simultaneously detected by DRI_uav at the moderate and severe drought stages (Table S9). Furthermore, only three and four loci were detected by both PWC_uav_R and LRS_uav, and both LRS_uav and DRI_uav, respectively; but no common locus was detected by DRI_uav and PWC_uav_R (Fig. 6a). Hence, the combination of LRS_uav and DRI_uav can be

more efficient to dissect the genetic basis of dynamic drought responses.

In addition, when compared with LRS_uav and PWC_uav_R, more stable loci (detected in multiple time points) and drought resistance-related genes were identified by the comprehensive DRI_uav indicator than the other two UAV traits. Among the 111 significant association signals detected by UAV traits during drought stress treatment, 17 loci were identified at two time points at least or by multiple traits. Three DRI_uav-associated loci (Locus 43, Locus 49 and Locus 67) were detected at three time points at least, and the P -values were 1.58×10^{-12} , 3.9×10^{-09} , and 1.6×10^{-09} , respectively. Locus 72 and Locus 109 ($P = 4.6 \times 10^{-08}$ and 3.8×10^{-08} , respectively) were identified at two time points by DRI_uav, one time point by LRS_uav, and two time points by PWC_uav_R (Table S9). Locus 42 was associated with DRI_uav for at least two time points, and *OsSRO1c* in this locus is significantly associated with drought resistance. Based on significant *OsSRO1c* promoter variation (Fig. 7a), two *OsSRO1c* haplotype groups showed significant differences in DRI_uav and relative expression levels (Fig. 7b,c). These data reveal how *OsSRO1c* participates in the rice drought response and improves drought resistance by exploring the function of drought resistance alleles in natural germplasm. In a previous GWAS of drought tolerance using a complete set of image traits and traditional traits in a population, including all the accessions in this study (Guo *et al.*, 2018), the loci containing *OsSRO1c* was not detected. As *OsSRO1c* confers drought response by promoting H₂O₂-mediated stomatal closure, which occurs at the early leaf-rolling stages to avoid severe water loss, it is reasonable that this locus was not detected in the previous study in which the image traits (such as green leaf area ratio and plant architecture features) were extracted at severe drought stress stages. Besides the loci containing reported drought resistance genes, most of the significant loci detected by UAV traits were not detected in an association study of glasshouse drought tolerance (Guo *et al.*, 2018) or other linkage mapping studies, suggesting that UAV-based field phenotyping is not only a complementary approach to the glasshouse-based phenotyping platforms, but is also very important for completely dissecting the genetic basis of the dynamic crop response under field drought conditions. More importantly, GWAS of dynamic traits with high temporal resolution (Knoch *et al.*, 2020; Li *et al.*, 2020b) may be valuable in determining key phenotyping stages during drought stress treatment in the field and thus detecting more important loci for drought resistance improvement.

Conclusions

The proposed UAV-RGB field phenotyping method has several advantages. Compared with imaging chambers and expensive UGV platforms, the UAV platform is more flexible and efficient to deploy for high-throughput phenotyping under field conditions, and its timeliness guarantees the reliability of phenotypic traits for large germplasm populations. Compared with spectral sensors, the cost-effective RGB camera is more stable in terms of acquiring high-resolution images and needs less processing such as reflectance calibration, which has strict requirements of stable illumination.

Besides, the robust deep learning model (DCNN-LRS) makes it possible to monitor the dynamic response to drought in the field precisely, regardless of the complex environmental factors. In this study, the application of UAV-RGB phenotyping has confirmed its effectiveness and the importance of dynamic UAV traits for detecting stable loci in key time windows during progressively developed drought stress treatment. Our results also show that combining GWAS with the dynamic UAV traits has advantages in exploring significant alleles in natural populations that may be beneficial for drought resistance breeding. Furthermore, the UAV-RGB phenotyping method can be extended to assess other crops for drought phenotype response in the field or other stresses which also cause leaf-rolling and water-loss phenotypes, thus accelerating stress-resistance crop breeding under complex field conditions.

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Author contributions

ZJ and HT designed the research, conducted the experiments and wrote the manuscript. ZJ collected the UAV data, performed the image analysis and UAV phenotypic traits extraction. HT conducted the field experiments and performed the GWAS analysis. BB provided the rice accessions and participated in manual phenotyping in 2019 with QL. CY advised on UAV remote sensing and revised the manuscript. BZ and ZG participated in the UAV data acquisition. HZ provided the raw genotype data. WY advised on plant phenomics. LX and JZ designed the research, supervised the project and wrote the manuscript. ZJ and HT contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Illustration of manual leaf-rolling rating and sample patches for different degrees of severity.

Fig. S2 Performance evaluation of DCNN-LRS.

Fig. S3 Histogram of brightness distribution of the image patches in the 2019 test dataset.

Fig. S4 Genetic variations of *OsbZIP12* and *OsRCI-25* were significantly associated with DRI_uav_D20_PM of rice at the reproductive stage under field conditions.

Notes S1 Visual leaf-rolling rating and plant water content measurements.

Notes S2 Configuration information of the imaging platform.

Notes S3 GCP management and georeferencing process in Agisoft PHOTOSCAN.

Notes S4 Image data augmentation.

Notes S5 Website to download training and validation datasets and the DCNN-LRS model.

Notes S6 Examples of reported genes with associated by DRI_uav_D20_PM.

Table S1 Information on the association population used in 2018.

Table S2 Information on the testing population used in 2019.

Table S3 Manually measured biomass and plant water content in 2019 for empirical regression based on the simultaneously measured AGV_uav trait.

Table S4 UAV image data acquisition time points in 2018.

Table S5 Manual LRS_m of each image patch in 2018 for DCNN-LRS model building.

Table S6 Manual LRS_m in 2019 for DCNN-LRS model testing.

Table S7 DCNN-LRS prediction accuracies for both accession replications in 2018.

Table S8 UAV traits for the 240 rice accessions used in the GWAS analysis.

Table S9 Information on the association loci.

Table S10 GWAS loci overlapped with previous reported QTLs.

Table S11 Expression levels of *OsSRO1c* in different haplotype groups under normal and drought stress in reproductive stage.

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