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Knockouts of Drought Sensitive Genes Improve Rice Grain Yield under both Drought and Well-watered Field Conditions

Guihua Lu[1*], Changgui Wang[2], Guokui Wang[2], Guanfan Mao[2], Jeffrey E Habben[1], Guangwu Chen[2], Min Liu[2], Yanlong Shi[2], Wei Wang[2], Xiping Wang[2], Huiting Li[2], Yang Gao[2], Pingping Qu[2], Hua Mo[1], Mary K. Beatty[1], H. Renee Lafitte[1], Michael W Lassner[1], Richard M Broglie[1], Junhua Liu[2*], and Thomas W. Greene[1*]

[1]*Corteva Agriscience, Trait Discovery, Johnston, Iowa, IA 50131, USA*

[2]*Sinobioway Bio-Agriculture Group, Co., Ltd., Beijing 100085, China*

[*]**Corresponding authors:** Guihua Lu, Corteva Agriscience, Trait Discovery, Johnston, Iowa, IA 50131, USA, Tel: +0018582909312; E-mail: lug50131@yahoo.com

Junhua Liu, Sinobioway Bio-Agriculture Group, Co., Ltd., Beiing 100085, China, Tel: +8613521645063; E-mail: junhua.liu@sinobiowayagr.com

Thomas W Greene, Corteva Agriscience, Trait Discovery, Johnston, Iowa, IA 50131, USA, Tel: +0015159545949; E-mail: tom.greene@corteva.com

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Abstract

Rice (*Oryza sativa*) is one of the most important staple food crops feeding more than half of the world's population. One of the requirements for future sustainable rice production is to develop drought tolerant varieties. We have identified a number of drought sensitive tagged lines by screening our rice activation tagging population. Two of the sensitive lines, AH13391 and AH17392, exhibit reduced drought tolerance compared to the controls, and have a single T-DNA in a region next to an ATPase-associated with diverse cellular activities (AAA)-like gene, respectively. Constitutive overexpression of either AAA-like gene (OsAAA-1 and OsAAA-2) significantly reduced the drought tolerance, whereas knocking them out by CRISPR-Cas9 significantly increased grain yield under both drought and well-watered field conditions. Comparative analysis of different OsAAA-1-edited variations shows that the core AAA ATPase domain and the C-terminal end of OsAAA-1 protein are important for its function in drought sensitivity; and OsAAA genes may regulate drought sensitivity through interacting with other drought-stress responsive partners. Our results show that OsAAA genes play an important role in drought sensitivity and demonstrate the feasibility of improving drought tolerance by CRISPR-mediated knockouts of native rice drought sensitive genes.

Keywords: CRISPR-Cas9; Drought sensitivity; Drought tolerance; Grain yield; Rice

Introduction

Rice is one of the most important staple foods to meet the increasing food demand and the world population growth [1]. However, its sustainable production is facing challenges, including resource competition such as water and land, climate changes, and farm labor shortage and rising cost of production [2,3]. One of the potential solutions to solve these challenges is direct seedling of rice (DSR) which refers to the process of establishing a rice crop from seeds sown in the field rather than by transplanting seedlings from the nursery [4]. This practice eliminates the laborious process of planting seedlings by hand and it substantially reduces the crop's water requirements [4]. On the other hand, the optimized DSR technology requires drought and herbicide tolerant traits for maintaining high yield under the new rice cultivation practice.

Drought stress remains the single most important factor that limits crop productivity worldwide [5-9]. Under a water-limiting environment, plants undergo a cascade of molecular, biochemical, physiological, morphological, and developmental changes [7,10-12]. Although many reports on molecular mechanisms and genetic regulatory networks of drought tolerance have been published [6,11,13-18]. It remains a major challenge to fully understand the basic biochemical and molecular mechanisms for drought perception, transduction, tolerance, and resistance. Genetic research has revealed that drought tolerance is controlled by many genes, and some drought tolerance genes, such as DREB and AREB/ABF TFs, have been discovered [11,19]. Molecular marker-assisted breeding and precision phenotyping have led to improved drought tolerance in corn [20,21]. However, marker accuracy and breeding efficiency remain problematic [22]. Transgenic approaches to engineering drought tolerance in crops have made some progress [11,16,19,23,24]. Although drought response and resistance have been extensively studied and various technologies have been applied in developing drought tolerant crops, no successful commercial products have been developed.

The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 (CRISPR-associated protein 9) genome editing technology has shown great promise for quickly addressing emerging challenges in agriculture [25,26]. It can be used to precisely edit plant genome sequences to achieve the desired traits, and has significant advantages compared to other genome editing technologies [1,25]. Application of CRISPR-Cas9 technology in editing the plant genome for improving plant protection and abiotic stress tolerance has made remarkable progress [1,8,9,25,26]. CRISPR-Cas9-induced mutagenesis of Semi-Rolled Leaf1,2 confers curled leaf phenotype and drought tolerance in rice [27]. CRISPR-Cas9-mediated knockouts of OsSAPK2, SlMAPK3, and SlNPR1 resulted in reduced drought tolerance in tomato and rice plants [28-30]. The novel alleles for OsT2/AHA1 mutant generated by CRISPR-Cas9 exhibited enhanced stomatal responses in Arabidopsis [31]. Shi et al. demonstrated that CRISPR-Cas9 induced ARGOS8 variations showed improved maize grain yield under field drought conditions, revealing the potential of CRISPR-

Cas9 system for creating novel allelic variations for developing drought tolerant crops [32].

BCS1 (cytochrome bc1 synthase1) is a transmembrane chaperone found in the mitochondrial inner membrane in yeast and mammalian. It is required for the respiratory chain complex III assembly which plays an important role in transferring electrons from the Rieskeiron-sulfur protein to cytochrome c [33]. BCS1 contains a C-terminal AAA ATPase domain in the matrix side that is essential for chaperone function [34]. AAA ATPase was first defined as 'ATPase Associated with diverse cellular activities' and conserved in prokaryotes and eukaryotes [35]. BCS1-like genes have been identified and characterized as one of the stress-responsive nuclear genes encoding mitochondria and chloroplast proteins in dicotylous plants, and are located on the mitochondrial outer membrane and affects cell death and resistance to abiotic and biotic stresses [36-41]. Based on its outer mitochondrial membrane location and lack of BCS1 domain as well as the protein size, Zhang et al. re-annotated AtBCS1(At3g50930) as AtOM66 (Outer Mitochondrial membrane protein of 66 kDa) [41].

By screening our rice activation tagging population, we have identified tagged lines exhibiting enhanced drought tolerance and increased drought sensitivity [42]. Two of the recapitulated drought sensitive genes are ATPase associated with diverse cellular activities (AAA)-like genes, named as OsAAA-1 and OsAAA-2, respectively. We demonstrated that overexpression of either OsAAA-1 or OsAAA-2, significantly reduced drought tolerance, whereas knockouts of them significantly increased drought tolerance in rice under both drought stress and well-watered field conditions. These results demonstrate that OsAAA genes play important roles in plant drought stress biology, and it is feasible to improve drought tolerance by knocking out drought sensitive genes via CRISPR-Cas9 technology towards developing drought tolerant rice varieties.

Materials and Methods

Plant materials and transformation

The rice activation tagging population was developed using a four-tandem copy of the enhancer of Cauliflower mosaic virus (CaMV) 35S promoter as described previously [42]. Rice transformation essentially followed the Agrobacterium-mediated method as described by Lin and Zhang [43]. The transgenic seedlings (T_0) were rescued and T_1 seeds were further advanced to the T_2 and T_3 generations in Beijing field (40°09′ N, 116°19′ E), and then stored at 4 °C. The tissue-culture events derived from wild type Zhonghua 11 (named as ZH11-TC and has no exogenous DNA transformed),events developed from empty vector DP0158 (Supplemental Figure S1A) were used as controls in greenhouse assays; the null seeds from the corresponding testing events were DsRED-negative and non-transgenic based on fluoresce and PCR-tests and were used as controls for testing overexpression and RNAi constructs; and seeds of non-edited lines with wild type OsAAA-1 or OsAAA-2 gene from transformations with DP2317, DP2354, or DP2805 were used as controls in various field experiments.

Materials reported in this paper may be subject to third party ownership and/or to governmental regulations. Availability of materials reported in this paper to academic investigators for non-commercial research purposes under an applicable material transfer agreement will be subject to the requisite permission from any third-party owners of all or parts of the materials and to governmental regulation considerations. Obtaining the applicable permission from such third-party owners will be the responsibility of the requestor. Transgenic materials and genome edited materials reported in this paper would only be made available if in full accordance with all applicable governmental regulations and the recipient's agreement to comply with applicable stewardship requirements.

Greenhouse drought assays

The greenhouse drought tolerance assays were conducted as described in Lu et al. [42].

Field drought assay and data analyses

For the drought assays of mature rice plants at Hainan field (18°25′ N, 108°58′ E) and Ningxia field (38°34′ N, 106°22′ E) of China, 10-15 transgenic events from each gene construct were tested. The T_2 or T_3 seeds were first sterilized as described in the greenhouse assay [42]. The germinated seeds were planted in a seedbed field. At 3-leaf stage, the seedlings were transplanted into the testing field, with 4 replicates and 10 plants per replicate for each transgenic event or knockout lines, and the 4 replicates were planted in the same block. T_2 or T_3seeds of non-edited lines with wild type OsAAA-1 or OsAAA-2 gene from transformations with DP2317, DP2354, or DP2805 were planted in the same block and used as controls in various experiments except noticed for the experiments. The rice plants were managed by normal practice using pesticides and fertilizers. Watering was stopped at the panicle initiation stage 2, so that to give drought stress at flowering and grain-filling stages with <20% of soil water volumetric content depending on the weather conditions (temperature and humidity). The soil water volumetric content was measured every 4 days at about 5 sites per block using TDR300 (Spectrum Technologies, Inc.). Plant phenotypes were observed and recorded during the experiments. The phenotypes include heading date, leaf rolling degree, drought sensitivity and drought tolerance. Special attention was paid to leaf rolling degree at noontime. At the end of the growing season, six representative plants were harvested from the middle of the row per line, and grain weight per plant was measured and calculated as a grain yield parameter for statistical analysis.

The field experimental design was set up as nested design in which event is nested within construct. To analyze the data, a mixed model framework was used to perform the single-location analysis. The yield data were statistically analyzed using mixed linear model by ASREML (VSN International Ltd), and the values are BLUEs (Best Linear Unbiased Estimates [44,45]. In the single-location analysis, main effects of construct and event are considered as a fixed effect. The blocking factors such as replicates, and field spatial variation are considered as random. There are 3 components of spatial effects including row, column and autoregressive correlation as AR1*AR1 which are included to reduce noise caused by spatial variation in the field. The significance test between event and controls was performed using a p-value of 0.05 in a two-tailed test. The outliers were declared if their standard deviation is above 4, and the outliers were removed from the analysis.

T-DNA-flanking sequence and identification of activation-tagged genes

To obtain the flanking sequence of AH13391, genomic DNA was isolated from 3-week-old seedling leaf tissues of the tagging lines using the Hi-DNA secure Plant Kit (TIANGEN, DP350) to identify the T-DNA flanking sequence by the plasmid rescue method [46]. Ten μg of

genomic DNA was digested overnight with 2 µL each of BglII/HindIII/XhoI (NEB, Ipswich, MA) in a 200 µl reaction solution. The self-ligation sample was transformed into competent E. coli DH5α via electroporation. The rescued clones were sequenced using the primer of M13R-46 and the right border-flanking sequences of the T-DNA were determined as previously described [47]. Based on the T-DNA insertion locus information, the T-DNA insertion site on both right border (RB) and left border (LB) were defined through PCR using a pair of primer complementing the T-DNA and the genomic DNA nearby the T-DNA insertion site, respectively. The flanking sequence of AH17392 was obtained by Southern-by-Sequencing [48].

OsAAA-1 and OsAAA-2 cloning and vector constructions

The cDNA of OsAAA-1 (LOC_Os05g51130) was cloned using primer GC-2208-F (5'-CTCACCCTCCCCATTCAACACTACTG-3') and primer GC-2209-R (5'-CATTCTTGTTGTCATTGTTGTACTCCAC-3'), and pooled cDNA from leaf, stem and root tissues of Zhonghua 11 plants as a template. The DNA fragment was ligated with XcmI-digested vector pSce-T and confirmed by DNA sequencing. DP0196 was constructed by ligating the 1.6 Kb I-SceI and PI-SceI fragment with OsAAA-1 with I-SceI and PI-SceI digested-pCAMBIA1300-DsRed-35s-DsRed-HEIP, and the OsAAA-1 sequences and orientation in DP0196 were confirmed by sequencing. OsAAA-2 (LOC_Os01g42030) was amplified from Zhonghua11 cDNA pool using primer GC-7008-F (5'-CTGCTGAGGGTTTTCAGAGACGTACCAGAGCCAAC-3') and primer GC-7009-R (5'-CCGCTGAGGGCACACTGTTTAAGCATCATTATTTG-3'). An expected ~1.5-Kb PCR fragment was purified, digested by Nb.BbvCI and ligated with BstXI-digested pMD19GW-Adv.ccdB (GKI-401). DP0962 was produced by the gateway LR reaction of GKI-401 and pCAMBIA1300DsRed-35S-GW-Adv.ccdB, the nucleotide sequences and orientation of OsAAA-2 in DP0962 were confirmed by sequencing.

To make DP1200, three pairs of primers were designed: FRigc442-1 (5'-CTGCTGAGGTGATGTTGCACTCCTTG-3') and FRigc442-2 (5'-GCTTGCTGAGGCTAAGTGCATTCATCAGTTG-3') for cloning the target fragment in forward orientation; RRigc442-1 (5'-CCGCTGAGGTGATGTTGCACTCCTTG-3') and RRigc442-2 (5'-GCCTGCTGAGGCTAAGTGCATTCATCAGTTG-3') for cloning the target fragment in reverse orientation; and Intron-F (5'-GCAAGCTGAGGGTACGGACCGTACTACTCTATT-3') and Intron-R (5'-GCAGGCTGAGGCTATATAATTTAAGTGGAAAAAAAGG-3') for cloning the linker intron [49]. The RNAi construct DP1200 was obtained by the gateway LR reaction and pCAMBIA1300DsRed-35S-GW-Adv.ccdB, the nucleotide sequences and orientation of the target fragment and linker intron in DP1200 were confirmed by sequencing.

Construction of the genome editing-related vectors

The basic CRISPR-Cas9 binary vector was generated as described and the guiding RNAs were designed as previously described (Wang et al., 2020). The guiding RNAs to target OsAAA-1 and OsAAA-2 genes are: OsAAA-1-g5 (sgRNA-5), 5'-TCCCTGGTGGACCATACAGC-3'; OsAAA-1-g6 (sgRNA-6), 5'-AAAAGATGCCAGCGAGACCA-3'; OsAAA-2-g1(sgRNA-1), 5'-ACCTGAACTCGGAGCCCTCA-3'; OsAAA-2-g3 (sgRNA-3), 5'-ATCTGGGCTACTCAACTTCG-3'. The sgRNA target oligonucleotides were synthesized to create sticky ends as follows: Target-sense: 5'-CAG-gRNA sense; and Target-Anti: 5'-AAC-gRNA antisense.

Generation of the guiding RNA expression cassettes and construction of CRISPR-Cas9 binary vectors

The annealing products of target-sense and target-anti oligonucleotides were ligated with BspQI digested-pHSG396GW-URS-rU6DsRed&UC-mpCas9 for the targets sgRNA-5 and sgRNA-6 of OsAAA-1 and BspQI digested-pHSG396GW-URS-rU6DsRed for the targets sgRNA-5 of OsAAA-1. The guiding RNAs expression cassettes of OsAAA-2 were constructed as previously described [47]. To generate multiple genome editing vector with two guiding RNA expression cassettes, pHSG396GW-URS-rU6-OsAAA-1-g5 was digested by Nb.BsrDI to get expected size of ~3 kb product, the harvested digestion was ligated to make the intermediate multiplex vector containing guiding RNAs expression cassettes of pHSG396GW-URS-rU6-OsAAA-1-g5&3&UC-mpCas9, the construct was confirmed by PstI digestion and sequencing. The multiplex vector containing guiding RNAs expression cassettes of OsAAA-2-g1&3 was constructed as previously described [47]. The CRISPR-Cas9 binary vectors of OsAAA-1 and OsAAA-2 were created and confirmed by sequencing. The resulted CRISPR-Cas9 binary vectors were illustrated in Supplemental Figure S1.

RT-PCR analyses

The RT-PCR analyses were conducted as previously described [47]. The primers used in the RT-PCR analysis for OsAAA-1 and OsAAA-2 were as below: DP0196-F1：5'-CCTTGGTCTACTGGAGCTCC-3', DP0196-R1：5'-GTTCTCCATCGCTTTGCTATC-3'; DP1200-F1: 5'-GATTCTTGCCAGCAACTACCAC-3', DP1200-R1: 5'-CCAGTAGACCAAGGAGTGCAAC-3'; DP0962-F1: 5'-GGCAAAGCTGAAAATGTGGAG-3', DP0962-R1: 5'-GGGATCTTGATTCTCTGGGAC-3'.

Sequence alignments

Alignments of the DNA and protein sequences were performed using the Clustal V method with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10) [50].

Results

Identification of drought sensitive tagging lines AH13391 and AH17392

Drought stresses occurring at flowering and grain-filling stages have the greatest impact on crop production, therefore, we have developed a field drought screening method that targets these growth stages (Figures 1A-1F). In this assay, we stop watering the field at panicle initiation stage 2 which results in soil volumetric water content less than 20% at the rice flowering and grain-filling stages under natural conditions in Sanya (18°25′ N, 108°58′ E) of Hainan Province and Yinchuan (38°34′ N, 106°22′ E) of Ningxia Province, China. The plants received no further irrigation after the stage 2, unless rain fall occurred. The rice plants were harvested and grain yields were measured as described in the Materials and Methods. A number of drought tolerant and sensitive lines were identified by screening our rice activation tagging population in the fields. Two of the drought sensitive lines, AH13391 and AH17392, showed wilting leaves about two weeks after watering was stopped, and gradually leaf necrosis occurred in plants at both Hainan and Ningxia fields (Figures 1C and 1E). This phenotypic response was reproducible across locations and

years. Interestingly, both lines have a single T-DNA insertion locus near a mitochondrial chaperone BCS1-like gene (LOC_Os05g51130 named as OsAAA-1 and LOC_Os01g42030 named as OsAAA-2) next to the right border (RB) of the inserted T-DNA in the lines (Figures 1G and 1H) based on the rice genome database search (MSU RGAP Release 7). The qRT-PCR analyses indicate that OsAAA-1 transcript increased ~20-fold in the leaf tissues of AH13391, indicating the drought sensitive phenotype may be related to the activated expression of OsAAA-1 and OsAAA-2.

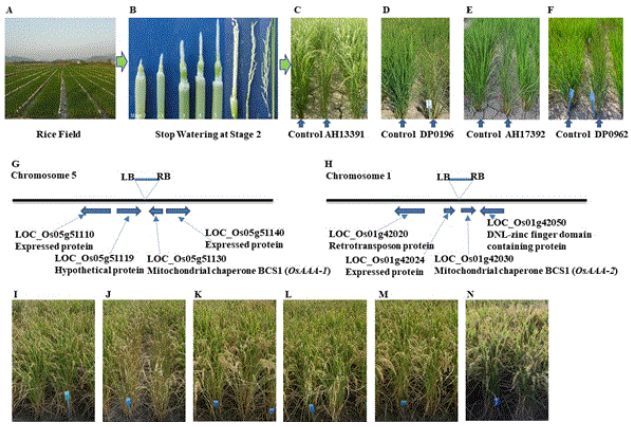

Figure 1: Field drought screening method and identifications of drought sensitive lines AH13391 and AH17392, and drought sensitive and tolerance events of DP0196, DP0962, DP1200, DP2317, DP2354, and DP2805. Seedlings were transplanted at 3-W-old into rice field (A), and then stop watering at panicle initiation stage 2 (B) to result in flowering and grain filling stage drought stress. The leaf wilting phenotypes of AH13391 (C) and AH17392 (E) were observed 2 weeks after stop watering. Map of the T-DNA insertion and the nearby genes in AH13391 and the right border (RB) at 29,268,293 bp of Chromosome 5 (G), and Map of the T-DNA insertion and the nearby genes in AH17392 and the right border (RB) at 23,832,505 bp of Chromosome 1 (H). The phenotypes of Control, DP0196, DP0962, DP1200, and DP2317, DP2354, and DP2805 events at vegetative stage (D and F) and mature stages (I-N). Control (C and E), ZH11-TC; Control (D and F), corresponding nulls; and Control (I), non-edited wild-type OsAAA-1. LB, left border.

Recapitulation of the drought sensitive genes

To recapitulate the drought sensitive genes from these drought sensitive lines, we cloned two candidate genes on the left boarder (LB) side and two candidate genes on the right border (RB) side of the T-DNA for each line, made overexpressing constructs with the genes being driven by the cauliflower mosaic virus 35S (CaMV35S) promoter, and transformed the constructs into Zhonghua 11. The field drought assays of the transgenic rice plants with these gene constructs showed that overexpression of OsAAA-1 (Figure 2A, Supplemental Figure S1B, DP0196) and OsAAA-2 (Figure 2B, Supplemental Figure S1C, DP0962), respectively, recapitulated the drought sensitive phenotypes of the corresponding tagged lines under field drought conditions (Figures 1D and 1F). The average grain yields of 22 DP0196-events and 12 DP0962-events were significantly reduced 44% (P=0.0081) and 58% (P=0.0068) compared to the control (the pooled null seeds from DP0196 or DP0962 events were used as the control in the separated experiments), respectively. Further analyses revealed that the drought sensitivity of the DP0196 events is closely related to the transgene expression level. The average yield of 3 high overexpressing events was decreased by 62%, whereas the average yield of the 3 low overexpressing events was reduced by 20% compared to the control (Supplemental Table S1). Leaf wilting phenotypes were associated with high overexpressing events, whereas the low overexpressing events did not show the leaf wilting phenotype at early stress period. Similar results with DP0962 plants (Table 1) were obtained under field conditions. These results clearly showed that OsAAA-1 and OsAAA-2 are drought sensitive genes.

Location	Line ID	Number of events	Yield (g/plant) (average ± SE)	Change	P value[c]
Hainan	Control-1[a]	-	4.45 ± 0.97	-	-
	DP0196(OX)	2	1.98 ± 1.07	-56%	0
	DP1200(RNAi)	2	6.75 ± 1.07	52%	0.0001
	Control-2[b]	-	3.93 ± 0.62	-	-
	DP0962(OX)	3	3.52 ± 0.63	-11%	0.539
Ningxia	Control-1	-	7.69 ± 1.14	-	-
	DP0196(OX)	2	4.29 ± 1.27	-44%	0.0016
	DP1200(RNAi)	3	9.73 ± 1.33	27%	0.0693
	Control-2	-	23.38 ± 1.03	-	-
	DP0962(OX)	4	10.21 ± 1.42	-56%	0

Note: [a] The null seeds from DP0196 and DP1200 were pooled and used as control-1.

[b] T_2 seeds of non-edited lines form transformation with DP2805 were used as control-2.

[c] Statistical analyses of yields compared to control; 4 replicates and 6 plants per replicate. OX, overexpression construct; and RNAi, RNA interference construct.

Table 1: Reduced and enhanced drought tolerance of DP0196-and DP1200-transgenic rice plants at T_2 generation under Hainan and Ningxia field drought conditions.

BLAST searches revealed that both OsAAA-1 and OsAAA-2 encode polypeptides which have high homology to the mitochondrial chaperone BSC1 in yeast and plants. OsAAA-1 and OsAAA-2 have 71% amino acid identity each other, and have 45% and 44% amino acid identity with AtOM66, respectively [41]. They have a core AAA ATPase domain within a P-loop containing nucleotide triphosphate hydrolase domain (Figures 2D and 2E, Supplemental Figure S2). Based on its outer mitochondrial membrane location and lack of BCS1 domain as well as the protein size, Zhang et al. re-annotated AtBCS1(At3g50930) as AtOM66 (Outer Mitochondrial membrane protein of 66kDa) [41]. OsAAA-1 and OsAAA-2 have molecular weights of 54.99 kDa and 55.48 kDa, respectively, and contain a conserved core AAA ATPase domain as AtOM66 (Supplemental Figure S2). Therefore, we re-annotated the rice mitochondrial chaperone BSC1-like genes as OsAAA-1 and OsAAA-2, respectively.

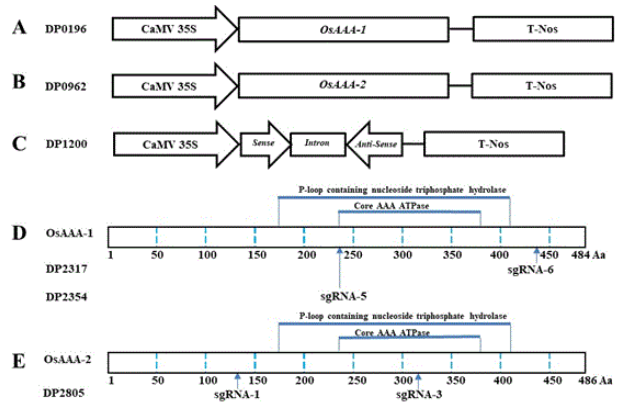

Figure 2: Schematic diagrams of DP0196 (A), DP0962 (B), DP1200 (C), DP2317 and DP2354 (D), DP2805 (E), and OsAAA-1 and AAA-2 protein structures with functional domains (D and E). CaMV 35S, cauliflower mosaic virus 35S promoter; OsAAA-1, rice AAA ATPase associated with diverse cellular activities (AAA)-1 protein; OsAAA-2, rice AAA ATPase associated with diverse cellular activities (AAA)-2 protein; T-Nos, Nos terminator; Intron, the first intron from potato GA20 oxidase gene (Gan et al., 2010); Sense and Anti-sense, the sense and antisense strands of the 163 bp fragment from OsAAA-1. Core AAA ATPase, AAA ATPase, core domain (246~381Aa) and P-loop containing nucleoside triphosphate hydrolase domain (179~434 Aa). DP2317 is designed to edit the OsAAA-1 using sgRNA-5 at 243 Aa and DP2354 is designed using sgRNA-6 at 453 aa for knockout of OsAAA-1; and DP2805 is designed using sgRNA-1 at 130 Aa and sgRNA-3 at 340 Aa for knocking OsAAA-2 out. Aa, amino acid residue. Arrowheads indicate direction of transcripts.

Downregulation of OsAAA-1 by RNAi increased drought tolerance

The low overexpressing DP0196 events have less drought sensitivity compared to the high overexpressing events (Supplemental Table S1). To understand if decreasing the endogenous level of OsAAA-1 mRNA can increase drought tolerance, an OsAAA-1 specific RNAi construct (Figure 2C, Supplemental Figure S1D, DP1200) was made and tested. Based on primary screening results, we selected two strong overexpressing DP0196 events and 2-3 strong silenced DP1200 events and tested them in both Hainan and Ningxia fields. As shown in Table 1, downregulation of the OsAAA-1 by RNAi (reduction of the steady-state level of endogenous OsAAA-1 mRNA by ~80% based on qRT-PCR analyses) significantly increased the grain yield under field drought conditions at both locations.

Greenhouse 3-leaf-stage drought assays showed that overexpression of OsAAA-1 (DP0196) significantly reduced survival rate >30%, whereas silencing OsAAA-1 by RNAi (DP1200) significantly increased survival rate >10% compared to ZH11-TC (the tissue-culture events derived from wild type Zhonghua 11 and has no exogenous DNA transformed) and DP0158 (Supplemental Figure S1A) controls (Supplemental Table S2). The greenhouse and field drought tolerance assays showed that there were no statistical differences among ZH11-TC, DP0158-transgenic events, and DP0196-event nulls (data not shown). These results are consistent with the field data, indicating that silencing the OsAAA-1 gene can increase drought tolerance not only at reproductive stages, but also seedling vegetative growth stages (Table 1).

Knockout of OsAAA-1 by CRISPR-Cas9 increased grain yield under drought and well-watered field conditions

To understand if completely knocking out the OsAAA-1 gene can further increase drought tolerance, we designed and made two CRISPR-Cas9 constructs, DP2317 and DP2354, to create OsAAA-1 allelic variants (Figure 2D, Supplemental Figures S1E and S1F).

These two constructs were transformed into Zhonghua 11, analyzed, and tested. The primers were designed to amplify the target sequences near the genome targeting sites using the genomic DNA of the transgenic rice plants at T_0, T_1, or T_2 generations as templates. The amplified target fragments were sequenced to confirm the edited results, which are shown in Supplemental Figure S3 and Supplemental Figure S4. Mutations such as insertion, deletion, or substitution of at least one nucleotide were produced, which resulted in the early termination of the coding sequence, translation shift and/or deletion of at least one amino acid residue. As shown in Supplemental Figure S3, there are 6 variations produced at sgRNA-6 site in DP2317 rice plants. Three mutants resulted in a translation shift, and have about 30 new amino acid residues at the C-terminal end and have similar protein length as the wild type OsAAA-1 protein; the other three mutants resulted in early stops of the open reading frame and polypeptides of 450 to 454 amino acid residues in length, about 30 amino acid residues shorter than the wild type OsAAA-1 protein. There are 12 edited variants produced at sgRNA-5 site in DP2354 rice plants. All these 12 mutants resulted in early termination of translation. The translated polypeptides have 244 to 284 amino acid residues in length, lacking the core AAA ATPase domain and having a partial P-loop (Supplemental Figure S4).

Downregulation of OsAAA-1 by RNAi and CRISPR-Cas9 did not result in any visible abnormal phenotypes including plant height, flowering time, and maturity under well water and drought conditions, but apparently higher yields than control (Figures 1I-1N).

We evaluated the events of these constructs side-by-side in our Hainan and Ningxia fields using non-edited plants as controls. As indicated in Table 2, DP2317 plants from 6 homozygous lines significantly increased grain yield under drought conditions, and had no yield penalty under well-watered conditions in Hainan field. DP2354 plants from 12 homozygous lines significantly increased grain yield under drought conditions in both Hainan and Ningxia fields, and slightly increased yield under well-watered conditions compared to the non-edited control. The control plants reduced yield by 84% and 28% under drought conditions compared to well-watered conditions in Hainan and Ningxia fields, respectively, indicating the drought stress levels were significantly different between the two locations. These facts demonstrated that down-regulation of OsAAA-1 by RNAi and CRISPR can increased rice grain yield under a relatively broad range of drought stress levels.

DP2354 was designed to eliminate the core AAA ATPase domains and truncate the P-loop containing triphosphate hydroxylase domain, while DP2317 was designed to keep these two domains. DP2354 increased drought tolerance significantly higher than DP2317 in Hainan experiments, indicating that these two domains contributed to drought sensitivity.

Location	Treatment	Line ID	Number of events	Yield (g/plant) (average ± SE)	Change	P value[d]
Hainan	Drought	Control [a]	-	3.91 ± 0.38	-	-
		DP2317(CRISPR)[b]	6	4.66 ± 0.35	18%	0.0488
		DP2354(CRISPR)[c]	12	5.16 ± 0.35	30%	0.001
	Well-Water	Control	-	23.75 ± 0.69	-	-
		DP2317(CRISPR)	6	24.52 ± 0.65	3%	0.3362
		DP2354(CRISPR)	12	25.21 ± 0.62	6%	0.0654
Ningxia	Drought	Control	-	23.38 ± 1.03	-	-
		DP2354(CRISPR)	12	28.67 ± 1.52	23%	0.0012
	Well-Water	Control	-	32.62 ± 1.13	-	-
		DP2354(CRISPR)	12	34.21 ± 1.87	5%	0.4172

Note: [a] T_2 or T_3 seeds of non-edited lines from transformations with DP2317 and DP2354 were used as control. [b] 6 DP2317-edited homozygous lines (Supplemental Figure S3). [c] 12 DP2354-edited homozygous lines (Supplemental Figure S4) were used in the experiments. [d] Statistical analyses of yields compared to control; 4 replicates and 6 plants per replicate. T_3 seeds were used in Hainan field experiments, and T_2 seeds were used in Ningxia field experiments. CRISPR, CRISPR-Cas9 construct.

Table 2: Enhanced grain yields of DP2317- and DP2354-edited rice plants at T_2 and T_3 generations under drought and well-watered conditions at Hainan and Ningxia fields.

The C-terminal 30-amino acid domain is important for the function of OsAAA-1

DP2317 plants significantly increased drought tolerance compared to non-edited control (Table 2). Further analyses revealed that early translation stop mutant lines DP2317(E) increased drought tolerance significantly more than late translation stop lines DP2317(L) under both drought and well-watered field conditions (Table 3, $P<0.01$). The DP2317 (E) plants contain a mutant OsAAA-1 protein which lacks ~30 amino acid residues at its C-terminal end, whereas DP2317(L) rice plants have a mutant OsAAA-1 protein with 30 new amino acid residues at its C-terminal end (Supplemental Figure S3). The mutant OsAAA-1 protein sequence in DP2317 (L) plants have limited homology with wild type OsAAA-1 protein at the C-terminal end (Supplemental Figure S3). These results indicate that different edited variations can have different functions. These 30 amino acid residues at the C-end of wild type OsAAA-1 and DP2317 (L) have a theoretical isoelectric point of 4.15 and 13.29 by Serial Cloner, respectively, indicating that lack of this C-end peptide or change it into an alkalic peptide may impact the three-dimensional structure and configuration of the OsAAA-1 protein as well as its function in drought sensitivity.

Location	Treatment	ID	Number of events	Yield (g/plant) (average ± SE)	Change	P value[e]	P value[f]
Hainan-17	Drought	Control[a]		5.21 ± 0.60			-
		DP2317(CRISPR)[b]	6	7.68 ± 0.55	47%	0	-
		DP2317 (L)[c]	3	6.35 ± 0.61	22%	0.0388	-
		DP2317 (E)[d]	3	9.00 ± 0.62	73%	0	0
Hainan-18	Drought	Control		3.91 ± 0.38			-
		DP2317(CRISPR)	6	4.66 ± 0.35	18%	0.0488	-
		DP2317 (L)	3	3.86 ± 0.42	-3%	0.9128	-
		DP2317 (E)	3	5.46 ± 0.42	38%	0.0007	0.0005
Hainan-18	Well-water	Control		23.75 ± 0.69			-
		DP2317(CRISPR)	6	24.52 ± 0.65	3%	0.3362	-
		DP2317 (L)	3	22.84 ± 0.87	-4%	0.3584	-

		DP2317 (E)	3	26.21 ± 0.83	10%	0.0101	0.0022

Note: [a] T_2 or T_3 seeds of non-edited lines from transformations with DP2317 were used as control. [b] 6 DP2317-edited homozygous lines were used in the experiments as showed in Supplemental Figure S3. [c] DP2317 (E), 3 early translation terminated lines (DP2317P.05B.24, DP2317P.11B.05, and DP2317P.11B.28). [d] DP2317 (L), 3 late translation terminated lines (DP2317P.01B.01, DP2317P.02B.05, and DP2317P.03B.01). [e] statistical analyses of yields compared to control; 4 replicates and 6 plants per replicate. [f] Yield statistical analyses of DP2317(L) compared to DP2317(E). T_2 seeds were used in Hainan-17 experiments, and T_3 seeds were used in Hainan-18 experiments. CRISPR, CRISPR-Cas9 construct.

Table 3: Enhanced and reduced grain yields of DP2317-edited rice lines at T_2 and T_3 generations under drought and well-watered conditions at Hainan field.

Knockouts of OsAAA-2 increased grain yield under both drought and well-watered field conditions

OsAAA-2 has 71% amino acid identity with OsAAA-1. To cross-validate the results obtained with OsAAA-1, we designed and made a CRISPR-Cas9 construct DP2805 (Figure 2E and Supplemental Figure S1G). The DP2805 edited variations are shown in Supplemental Figure S5. Mutations such as insertion, deletion or substitution of at least one nucleotide were produced, which resulted in the early termination of the coding sequence, translation shift and/or deletion of at least one amino acid residue. As shown in Supplemental Figure S5B, 7 variations produced at sgRNA-1 site in DP2805 rice plants have about 35 new amino acid residues at the C-terminal end, and another edited line had 72 new amino acid residues at its C-terminal end. These edited lines miss the core AAA ATPase domain and the P-loop domains. Ten mutations produced at the sgRNA-3 site were obtained (Supplemental Figure S5D) and all of them have various amino acid deletions. Six of the edited lines have a polypeptide of 339 to 386 amino acid residues in length and partial core AAA ATPase and partial P-loop domains (Supplemental Figure S5D). No completed deletion mutants between sgRNA-1 and sgRNA-3 were obtained.

As shown in Table 4, knockouts of OsAAA-2 (DP2805) significantly increased yield under both drought and well-watered conditions at both testing locations. DP2805-sgRNA-3 edited variations increased grain yield under drought stress conditions a little more than DP2805-sgRNA-1 (Table 4, Figure 2E, Supplemental Figure S5B).Interestingly, lines DP2805P.07B.13 and DP2805P.07B.18 were only missing " GLLNFVD " 7 amino acid residues at positions 334-340 (Supplemental Figures S2 and S5D) compared to the wild type OsAAA-2. These 7 amino acid residues are in the core AAA ATPase domain and conserved among OsAAA-1, OsAAA-2, and AtOM66 (Supplemental Figure S2). These two lines increased yield under field drought conditions, suggesting that "GLLNFVD" in the core ATPase domain may be important for drought sensitivity (Table 4).

These results consistently demonstrated OsAAA-2 is also a drought sensitive gene and knockouts of OsAAA-2 can also increase drought tolerance. The cross-validated results from OsAAA-1 and OsAAA-2 clearly illustrate a new strategy to improve drought tolerance in rice by CRISPR technology.

Location	Treatment	ID	Number of events	Yield (g/plant) (average ± SE)	Change	P value[d]
Hainan	Drought	Control [a]	-	3.93 ± 0.62	-	-
		DP2805 (CRISPR)	8	4.88 ± 0.49	24%	0.0833
		DP2805-sgRNA-1[b]	3	4.43 ± 0.55	13%	0.4059
		DP2805-sgRNA-3[c]	5	5.15 ± 0.53	31%	0.0357
		DP2805P.07B.13	1	5.23 ± 0.79	33%	0.1028
	Well-water	Control	-	25.01 ± 0.96	-	-
		DP2805(CRISPR)	8	27.61 ± 0.90	10%	0.0044
		DP2805-sgRNA-1	3	28.42 ± 1.17	14%	0.0039
		DP2805-sgRNA-3	5	27.12 ± 1.03	8%	0.0444
Ningxia	Drought	Control	-	23.38 ± 1.03	-	-
		DP2805(CRISPR)	7	26.16 ± 1.07	12%	0.0253
		DP2805-sgRNA-1	3	25.80 ± 1.43	10%	0.1221
		DP2805-sgRNA-3	4	26.44 ± 1.32	13%	0.0375
		DP2805P.07B.13	1	25.49 ± 2.32	9%	0.3795
		DP2805P.07B.18	1	27.63 ± 2.32	18%	0.0781

	Well-water	Control	-	32.62 ± 1.13	-	-
		DP2805(CRISPR)	7	39.29 ± 1.28	20%	0
		DP2805-sgRNA1	3	39.51 ± 1.75	21%	0.0002
		DP2805-sgRNA3	4	39.13 ± 1.59	20%	0.0002

Note: [a] T_2 seeds of non-edited lines form transformation with DP2805 were used as control. [b] 7 DP2805-edited homozygous lines at sgRNA-1 site (Supplemental Figure S5A and S5B). [c] 10 DP2354-edited homozygous lines at sgRNA-3 site (Supplemental Figure S5C and S5D) were used in the experiments; and [d] statistical analyses of yields compared to control, 4 replicates and 6 plants per replicate. CRISPR, CRISPR-Cas9 construct.

Table 4: Enhanced grain yields of DP2805-edited rice plants at T_2 generation under drought and well-watered conditions at Hainan and Ningxia fields.

Predicted functional partners of OsAAA-1 and OsAAA-2 proteins

To understand the potential functional mechanisms of OsAAA-1 and OsAAA-2 chaperones, we searched their functional partners usingstring-db.org Database. As shown in Figure 3, there are 4 common partners between OsAAA-1 and OSAAA-2's partner groups, and they are ANK RF-domain containing protein (XBOS34); proteinase-like protein (OS06T0304600-02); ABC-domain containing protein (OS04T0660200-01); and novel protein (P0459B04.1). The first three classes of proteins are related to abiotic stress responses based on the published results [51-53]. Ankyrin repeat domain C3HC4-Type RING Finger Gene Family (XBOS34) is the first top partner for both OsAAA-1 and OsAAA-2. This family genes play important roles in plant growth and development as well as in stress responses [52]. XBOS34 encoded by LOC_Os07g26490 has been identified from a drought sensitive rice tagging line AH13610 in our research, AH13610 exhibited drought sensitive phenotype in repeated field assays, and XBOS34 is one of the candidates near by the T-DNA insertion locus in AH13610 (data not shown). These data further show that OsAAA genes are negative regulators of drought tolerance, and they may regulate drought sensitivity through interacting with other drought-stress responsive partners.

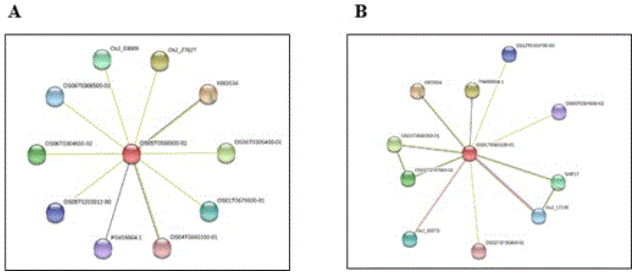

Figure 3: Predicted functional partners of OsAAA-1 (A) and OsAAA-2 (B) proteins revealed by string-db.org database.

A, OsAAA-1's predicted functional partners. OS05T0588900-01: OsAAA-1, LOC_Os05g51130 protein; XBOS34: LOC_Os07g26490 protein, probable E3 ubiquitin-protein ligase XBOS34 (513 aa); ankyrin repeat domain C3HC4-Type RING Finger Gene Family; OsJ_27627: LOC_Os08g36530.1 protein, aspartic proteinase nepenthesin precursor; OS06T0305400-01: LOC_Os06g20110 protein, expressed protein; OS06T0304600-02: LOC_Os06g20040 protein, aspartic proteinase nepenthesin-2; OsJ_03009: LOC_Os01g48750 protein, aspartic proteinase nepenthesin-2; OS01T0679500-01: LOC_Os01g48740 protein, aspartyl protease family protein; OS06T0306500-00: LOC_Os06g20190 protein, aspartic proteinase nepenthesin-2; OS05T0203912-00: LOC_Os09g20730 protein, peptidase aspartic family protein; P0459B04.1: LOC_Os01g74250 protein, plant-specific domain TIGR01615 family; OS04T0660200-01: LOC_Os04g56510 protein, ABC1 family domain containing protein. B, OsAAA-2's predicted functional partners. OS01T0605100-01: OsAAA-2, LOC_Os01g42030 protein; OS01T0747600-01: LOC_Os01g54380 protein, PPR repeat domain containing protein family; SAP17: LOC_Os09g21710 protein, AN1-like zinc finger domain containing protein; OsJ_15073: LOC_Os04g38860 protein, expressed protein; OsJ_12145: LOC_Os03g48080 protein, CHCH domain containing protein; OS12T0153700-00: LOC_Os12g05750 protein, eukaryotic aspartyl protease domain; OS02T0720900-01: LOC_Os02g48900 protein, aspartic proteinase nepenthesin-1.

Discussion

Drought stress is the most critical environmental factor which impacts crop productivity worldwide. We identified drought sensitive rice tagging lines by field screening of our rice activation tagging population and recapitulated the corresponding drought sensitive genes. Our study demonstrates that OsAAA-1 and OsAAA-2 are drought sensitive genes, and reducing their expression significantly increased drought tolerance in rice under field conditions. To our knowledge, this is the first report of increasing drought tolerance in plants by knocking out drought sensitive genes via CRISPR technology.

Overexpression of OsAAA-1 and OsAAA-2 significantly increase rice sensitivity to drought under field and greenhouse conditions (Table 1, Supplemental Tables S1 and S2), whereas downregulation of the OsAAA-1 gene expression by RNAi increased drought tolerance (Table 1). Furthermore, knockouts of the OsAAA-1 and OsAAA-2 by CRISPR-Cas9 significantly increased grain yield under field drought conditions with no yield drag under well-watered conditions. Under the water-limited environments at our two filed drought locations, control grain yields were reduced by >84% and 28% compared to well-watered conditions (Tables 2 and 4), respectively, indicating severe drought stress in Hainan field and moderate stress in Ningxia field. These results show that OsAAA-knockout rice lines improve drought tolerance under both moderate and severe drought stress conditions. In addition, knockout of these genes also increased grain yield under well-watered field conditions. No abnormal visible phenotypes were observed with the knockout lines during the rice growth and development stages under both drought and well-watered field conditions. The consistent results of downregulation of two OsAAA

genes by both RNAi and CRISPR technologies under two different field conditions, clearly demonstrated the feasibility of improving drought tolerance by editing rice drought sensitive genes.

Both OsAAA-1 and OsAAA-2 have a core AAA ATPase domain and a P-loop containing nucleotide triphosphate hydrolase domain (P-loop domain). DP2354 (without the Core AAA ATPase domain and with a truncate the P-loop domain) conferred higher yield than DP2317 (with the core AAA ATPase domain and P-loop domain) (Table 2). These results indicate the Core AAA ATPase and the P-loop domains contributed to the observed drought sensitivity with DP0196 (Table 1). Furthermore, lines DP2805P.07B.13 and DP2805P.07B.18 did not have the "GLLNFVD" amino acid residues at positions A334-340 (Supplemental Figure S5) compared to the wild type OsAAA-2. These 7 amino acid residues are within the core AAA ATPase domain and are conserved among OsAAA-1, OsAAA-2, and AtOM66 (Supplemental Figure S2), suggesting that "GLLNFVD" in the core ATPase domain is critical for the drought sensitivity (Table 4).

DP2317(E) rice plants showed significantly higher drought tolerance than DP2317(L) plants (Table 3). DP2317(E) lacks the 30-amino acid domain at the C-terminal end, whereas DP2317(L) has added about 30 new amino acid residues. The new C-terminal end of DP2317L has limited amino acid sequence homology with the wild type OsAAA-1 protein, except they have similar lengths of total amino acid residues (Supplemental Figure S3). These results indicate that the acidic C-terminal end of OsAAA-1 may be important in regulating drought sensitivity. The mammalian AAA ATPase BCS1 is a transmembrane chaperone found in the mitochondrial inner membrane, and its C-terminal AAA ATPase domain in the matrix side is essential for chaperone function [33-35]. The Arabidopsis AtOM66 has both the N-terminal and C-terminal are exposed on the outer mitochondrial membrane [41]. The C-terminal end of OsAAA-1 protein may be involved in mitochondrial localization, protein stability, membrane association, or signal transduction associated with drought sensitivity.

The AAA ATPase domain is associated with diverse cellular activities including membrane fusion, peroxisome biosynthesis, microtubule disassembly, and mitochondrial membrane protein complexes, ATP binding, and ATPase activity [54-57]. Under drought stress conditions, plants may become more conservative and save energy to withstand the stress period. However, overexpression of OsAAA-1 or OsAAA-2 in rice plants may waste ATP energy, whereas a knockout of the gene helps rice conserve energy. OsAAA-1 and OsAAA-2 are chaperones and they have several predicted functional partners which may be causative of the phenotypic responses (Figure 3). For example, ankyrin repeat domain C3HC4-Type RING Finger Gene Family (XBOS34) is the first top partner for both OsAAA-1 and OsAAA-2. This family genes play important roles in plant growth and development as well as in stress responses [52]. These possible mechanisms for explaining our observations need to be experimentally validated, but open a novel avenue for exploring drought sensitive mechanisms toward improving drought tolerance.

Zhang et al. reported that AtOM66 significantly increased drought tolerance, accelerate cell death, and amplifying salicylic acid signaling in Arabidopsis [41]. The rice results seem inconsistent with the Arabidopsis results in term of drought tolerance, and the inconsistence may be caused by the difference of their genetic backgrounds and/or difference in amino acid sequences among them since OsAAA-1 and OsAAA-2 have only 45% and 44% amino acid identity with AtOM66, respectively.

Conclusion

DSR technology has great potential for future sustainable rice production and drought tolerant rice varieties will greatly facilitate this approach. Combinations of genome editing, transgenic, and molecular breeding technologies can accelerate the development and cultivation of not only drought tolerance rice under a DSR management scheme, but also that of other crops.

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Author Contributions

G.L., C.W., and G.W. contributed equally. T.W.G. and G.L. conceived and supervised the project. G.L., T.W.G., C.W., G.W., J.L., J.E.H., W.W., X.W., H.L., M.B., R.L., M.W.L., and R.M.B. designed the experiments. G.M., G.C., M.L., Y.S., G.W., C.G., W.W., H.L., and G.Y. performed various experiments. P.Q., H.M., C.W., G.W., G.M., and J.L. analyzed the data. G.L., T.W.G., J.E.H., C.W., G.W., and J.L. wrote the manuscript with inputs from other authors. All authors have reviewed and approved of the manuscript prior to submission.

Accession Numbers

Accession numbers are as follows: OsAAA-1 (LOC_Os05g51130) and OsAAA-2 (LOC_Os01g42030) in the MSU RGAP Release 7.

Supplementary Information

Supplementary information is available at weblink

References

1. Mishra R, Joshi RK, Zhao K (2018) Genome editing in rice: recent advances, challenges, and future Implications. Front Plant Sci 9: 1361.
2. Zhang QF (2007) Strategies for developing green super rice. Proc Natl Acad Sci USA 104: 16402-16409.
3. Wassmann R, Jagadish SVK, Sumfleth K, Pathak H, Howell G, et al. (2009) Regional vulnerability of climate change impacts on Asian rice production and scope for adaptation. Adv Agron 102: 91-133.
4. Farooq M (2011) Rice direct seeding: Experiences, challenges and opportunities. Soil & Tillage Res 111: 87-98.
5. Boyer JS (1982) Plant productivity and environment. Sci 218: 443-448.
6. Chaves MM, Oliveira MM (2004) Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. J Exp Bot 55: 2365-84.
7. Kamanga RM, Mbega E, Ndakidemi P (2018) Drought tolerance mechanisms in plants: physiological responses associated with water deficit stress in Solanum lycopersicum. Adv Crop Sci Tech 6: 3.

8. Mushtaq M, Bhat JA, Mir ZA, Sakina A, et al. (2018) CRISPR/Cas approach: A new way of looking at plant-abiotic interactions. J Plant Physiol 224: 156-162.
9. Martignago D, Rico-Medina A, Blasco-Escámez D, Fontanet-Manzaneque JB, Caño-Delgado AI (2020) Drought Resistance by Engineering Plant Tissue-Specific Responses. Front Plant Sci.
10. Xoconostle-Cázares B, Ramírez-Ortega FA, Flores-Elenes L, Ruiz-Melrano R (2010) Drought tolerance in crop plant. Am J Plant Physiol 5: 241-256.
11. Hu H, Xiong L (2014) Genetic engineering and breeding of drought-resistant crops. Annual Rev Plant Biol 65: 715-741.
12. Butt YN, Fatima Q, Nasar S, Ikram J, Akram S (2017) Drought tolerance in plants: A Review. Int J Ecol Environ Sci 5: 19-28.
13. Xiong L, Zhu JK (2001) Abiotic stress signal transduction in plants: Molecular and genetic perspectives. Physiol Plant 112: 152-166.
14. Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. CurrOpin Plant Biol 6: 410-417.
15. Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta 218: 1-14.
16. Vinocur B, Altman A (2005) Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. CurrOpinBiotechnol 16: 123-132.
17. Yamaguchi-Shinozaki K, Shinozaki K (2005) Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters. Trends Plant Sci 10: 88-94.
18. Valliyodan B, Nguyen HT (2006) Understanding regulatory networks and engineering for enhanced drought tolerance in plants. CurrOpin Plant Biol 9: 189-195.
19. Nakashima K, Suenaga K (2017) Toward the genetic improvement of drought tolerance in crops. JARQ 51: 1-10.
20. Gaffney G, Schussler J, Löffler C, Cai W, et al. (2015) Industry-scale evaluation of maize hybrids selected for increased yield in drought-stress conditions of the US corn belt. Crop Sci 55: 1608-1618.
21. Adee E, Roozeboom K, Balboa GR, Schlegel A, Ciampitti IA (2016) Drought-tolerant corn hybrids yield more in drought-stressed environments with no penalty in non-stressed environments. Front Plant Sci 7: 1534.
22. Ashraf M (2015) Inducing drought tolerance in plants: recent advances. Biotechnol Adv 28: 169-183.
23. Castiglioni P, Warner D, Bensen RJ, Anstrom DC, Harrison J, et al. (2008) Bacterial RNA chaperones confer abiotic stress tolerance in plants and improved grain yield in maize under water-limited conditions. Plant Physiol 147: 455.
24. Lawlor DW (2013) Genetic engineering to improve plant performance under drought: physiological evaluation of achievements, limitations, and possibilities. J Exp Bot 64: 83-108.
25. Haque E, Taniguchi H, Hassan MM, Taniguchi H, Bhowmik P, et al. (2018) Application of CRISPR-Cas9 Genome Editing Technology for the Improvement of Crops Cultivated in Tropical Climates: Recent Progress, Prospects, and Challenges. Front Plant Sci 9: 617.
26. Jaganathan D, Ramasamy K, Sellamuthu G (2012) CRISPR for crop improvement: An Update Review. Front Plant Sci 9: 985.
27. Liao S, Qin X, Luo L, Han Y, Wang, X, et al. (2019) CRISPR/Cas9-induced mutagenesis of Semi-Rolled Leaf1,2 confers curled leaf phenotype and drought tolerance by influencing protein expression patterns and ROS scavenging in rice (Oryza sativa L.). Agronomy 9: 728.
28. Lou D, Wang H, Liang G (2017) OsSAPK2 confers abscisic acid sensitivity and tolerance to drought stress in rice. Front Plant Sci 8: 993.
29. Wang L, Chen L, Li R, Zhao R, Yang M, et al. (2017) Reduced Drought Tolerance by CRISPR-Cas9-Mediated SlMAPK3 Mutagenesis in Tomato Plants. J Agric Food Chem 65: 8674-8682.
30. Li R, Liu C, Zhao R, Wang L, Chen L, et al. (2019) CRISPR-Cas9-Mediated SlNPR1 mutagenesis reduces tomato plant drought tolerance. BMC Plant Biol 19: 38.
31. Osakabe Y, Watanabe T, Sugano SS, Ueta R, Ishihara R, et al. (2016) Optimization of CRISPR-Cas9 genome editing to modify abiotic stress responses in plants. Scientific Reports 6: 26685.
32. Shi J, Gao H, Wang H, Lafitte HR, Archibald RL, et al. (2017) ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. Plant Biotechnol 15: 207-216.
33. Tamai S, Iida H, Yokota S, Sayano T, Kiguchiya S, et al. (2008) Characterization of the mitochondrial protein LETM1, which maintains the mitochondrial tubular shapes and interacts with the AAA-ATPase BCS1L. J Cell Sci 121: 2588-2600.
34. Sawamura R, Ogura T, Esaki MA (2003) conserved α helix of Bcs1, a mitochondrial AAA chaperone, is required for the Respiratory Complex III maturation. Biochem Biophys Res Commun 443: 997-1002.
35. Erdmann R, Wiebel FF, Flessau A, Rytka J, Beyer A, et al. (1991) PAS1, a yeast gene required for peroxisome biogenesis, encodes a member of a novel family of putative ATPases. Cell 64: 499-510.
36. Sugimoto M, Yamaguchi Y, Nakamura K, Tatsumi Y, Sano H (2004) A hypersensitive response-induced ATPase associated with various cellular activities (AAA) protein from tobacco plants. Plant Mol Biol 56: 973-985.
37. Ho LH, Giraud E, Uggalla V, Lister R, Clifton R, et al. (2008) Identification of regulatory pathways controlling gene expression of stress-responsive mitochondrial proteins in Arabidopsis. Plant Physiol 147: 1858-1873.
38. Van Aken O, Whelan J (2012) Comparison of transcriptional changes to chloroplast and mitochondrial perturbations reveals common and specific responses in Arabidopsis. Front Plant Sci 3: 281.
39. Van Aken O, Zhang B, Carrie C, Uggalla V, Paynter E, et al. (2009) Defining the Mitochondrial Stress Response in Arabidopsis thaliana. Mol Plant 2: 1310-1324.
40. Van Aken O, Zhang B, Law S, Narsai R, Whelan J (2003) AtWRKY40 and AtWRKY63 Modulate the Expression of Stress-Responsive Nuclear Genes Encoding Mitochondrial and Chloroplast Proteins. Plant Physiol 162: 254-271.
41. Zhang B, Van Aken O, Thatcher L, De Clercq I, Duncan O, et al. (2014) The mitochondrial outer membrane AAA ATPase AtOM66 affects cell death and pathogen resistance in Arabidopsis thaliana. Plant J 80: 709-727.
42. Lu G, Wang X, Liu J, Yu K, Gao Y, et al. (2014) Application of T-DNA activation tagging to identify glutamate receptor-like genes that enhance drought tolerance in plants. Plant Cell Rep 33: 617-631.
43. Lin YJ, Zhang QF (2005) Optimising the tissue culture conditions for high efficiency transformation of Indica rice. Plant Cell Rep 23: 540-547.
44. Gilmour AR, Thompson R, Cullis BR (1995) Average information REML: an effective algorithm for variance parameter estimation in linear mixed models. Biometrics 51: 1440-1450.
45. Cullis B, Gogel B, Verbyla A, Thompson R (1998) Spatial Analysis of Multi-Environment Early Generation Variety Trials. Biometrics 54: 1-18.
46. Behringer FJ, Medford JI (1992) A plasmid rescue technique for the recovery of plant DNA disrupted by T-DNA insertion. Plant Mol Biol Rep 10: 190-198.
47. Wang C, Wang G, Gao Y, Lu G, Habben JE, et al. (2020) A cytokinin-activation enzyme-like gene improves grain yield under various field conditions in rice. Plant Mol Biol 102: 373-388.
48. Zastrow-Hayes GM, Lin H, Sigmund AL, Hoffman J, Alarcon C, et al. (2015) Southern-by-Sequencing: A Robust Screening Approach for Molecular Characterization of Genetically Modified Crops. The Plant Genome. pp: 10.
49. Gan D, Zhang J, Jiang H, Jiang T, Zhu S, Cheng B (2010) Bacterially expressed dsRNA protects maize against SCMV infection. Plant Cell Rep 29: 1261-1268.
50. Higgins DG, Sharp PM (1989) Fast and sensitive multiple sequence alignments on a microcomputer. Comput Appl Biosci 5: 151-153.

51. Yao X, Xiong W, Ye T, Wu Y (2012) Overexpression of the aspartic protease ASPG1 gene confers drought avoidance in Arabidopsis. J Exp Bot 63: 2579-2593.
52. Yuan X, Zhang S, Liu S, Yu M, Su H, et al. (2013) Global analysis of ankyrin repeat domain C3HC4-Type RING finger gene family in plants. PLoS ONE 8: e58003.
53. Ge K, Liu X, Li X, Hu B, Li L (2017) Isolation of an ABA transporter-like 1 gene from Arachis hypogaea that affects ABA import and reduces ABA sensitivity in Arabidopsis. Front Plant Sci 8: 1150.
54. Patel S, Latterich M (1998) The AAA team: related ATPases with diverse functions. Trends Cell Biol 8: 65-71.
55. Ogura T, Wilkinson AJ (2001) AAA+ superfamily ATPases: common structure-diverse function. Genes Cells 6: 575-597.
56. Iyer LM, Leipe DD, Koonin EV, Aravind L (2004) Evolutionary history and higher order classification of AAA+ ATPases. J Struct Biol 146: 11-31.
57. Hanson PI, Whiteheart SW (2005) AAA+ proteins: have engine, will work. Nat Rev Mol Cell Biol 6: 519-529.