Plant Cell Reports (2024) 43:207
https://doi.org/10.1007/s00299-024-03289-6

ORIGINAL ARTICLE

Cytokinin oxidase2-deficient mutants improve panicle and grain architecture through cytokinin accumulation and enhance drought tolerance in indica rice

Afreen Rashid[1,2] · V. Mohan M. Achary[1] · M. Z. Abdin[2] · Sangeetha Karippadakam[1] · Hemangini Parmar[1] · Varakumar Panditi[1] · Ganesan Prakash[3] · Pooja Bhatnagar-Mathur[4] · Malireddy K. Reddy[1]

Received: 26 March 2024 / Accepted: 16 July 2024 / Published online: 3 August 2024

Abstract

Key message **The *Osckx2* mutant accumulates cytokinin thereby enhancing panicle branching, grain yield, and drought tolerance, marked by improved survival rate, membrane integrity, and photosynthetic function.**

Abstract Cytokinins (CKs) are multifaceted hormones that regulate growth, development, and stress responses in plants. Cytokinins have been implicated in improved panicle architecture and grain yield; however, they are inactivated by the enzyme cytokinin oxidase (CKX). In this study, we developed a *cytokinin oxidase 2* (*Osckx2*)-deficient mutant using CRISPR/Cas9 gene editing in indica rice and assessed its function under water-deficit and salinity conditions. Loss of *OsCKX2* function increased grain number, secondary panicle branching, and overall grain yield through improved cytokinin content in the panicle tissue. Under drought conditions, the *Osckx2* mutant conserved more water and demonstrated improved water-saving traits. Through reduced transpiration, *Osckx2* mutants showed an improved survival response than the wild type to unset dehydration stress. Further, *Osckx2* maintained chloroplast and membrane integrity and showed significantly improved photosynthetic function under drought conditions through enhanced antioxidant protection systems. The *OsCKX2* function negatively affects panicle grain number and drought tolerance, with no discernible impact in response to salinity. The finding suggests the utility of the beneficial *Osckx2* allele in breeding to develop climate-resilient, high-yielding cultivars for future food security.

Keywords Grain yield · CRISPR/Cas9 · *ckx2* · Cytokinin · Drought tolerance · Rice

Introduction

Plant nutrient management to keep food production sustainable while ensuring environmental safety has become a major challenge in agriculture. Increasing fertilization use in agriculture to meet global food demands also has a cost to the environment. Hence, genetic enhancement of crop grain yield is a sustainable approach to meet the food needs of an ever-increasing population and to satisfy soil health (Bailey-Serres et al. 2015). Sink size (spikelets per unit land area), rate of grain filling, tiller number, and grain weight determine grain yield in rice (Mai et al. 2021). Plant reproductive architecture directly influences productivity and is crucial for developing high-yielding future crops. Thus, panicle architecture, which includes primary and secondary branching, is an important agronomic trait that is a key component of rice grain yield. Panicle density, which includes primary and secondary branching together,

Communicated by Ying hua su.

✉ V. Mohan M. Achary
vmmachary@gmail.com

M. Z. Abdin
mzabdin@jamiahamdard.ac.in

Malireddy K. Reddy
reddy@icgeb.res.in

[1] Crop Improvement Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, Delhi, India 110067

[2] Department of Biotechnology, Centre for Transgenic Plant Development, Jamia Hamdard, New Delhi, India 110062

[3] Plant Pathology, ICAR-Indian Agricultural Research Institute (IARI), New Delhi, India 110012

[4] Plant Breeding and Genetics, International Atomic Energy Agency (IAEA), PO-1001400 Vienna, Austria

determines grain productivity in rice (Huang et al. 2020). Genetic factors regulating grain number per panicle directly or indirectly, including *monoculm1* (*OsMOC1*), *grain number 1a/cytokinin oxidase 2* (*Gn1a/OsCKX2*), *erect panicle 3* (*OsEP3*), *dense and erect panicle 1* (*OsDEP1*), *panicle traits 2* (*OsPT2*), *squamosa promoter binding protein-like-14/ideal plant architecture 1* (*OsSPL14/OsIPA1*), *aberrant panicle organization 1* (*OsAPO1*), *large panicle* (*OsLP*), and *prostrate growth 1* (*OsPROG1*), have been well characterized in different rice (Lu et al. 2022; Yin et al. 2021).

The growth hormone cytokinins (CKs) have been implicated in both qualitative and quantitative components of crop yield. Cks regulates various important processes in plants, including cell division, differentiation, apical dominance, flower development, leaf senescence, stress response, and plant–pathogen interaction (Chen et al. 2020; Akhtar et al. 2020). Plants produce various types of CKs, including dihydrozeatin (DZ), N6-(D2-isopentenyl)-adenine (iP), trans-zeatin (tZ), and cis-zeatin (cZ). In wheat, about 20 different cytokinins exist. Among all the different forms, tZ is the most abundant in plants. The types of cytokinin and their activity differ among plant species. Different cytokinin molecules are active in plant tissues at different plant developmental stages and also under various environmental conditions (Chen et al. 2020; Akhtar et al. 2020). Cytokinin oxidase/dehydrogenase as the key enzyme maintaining Cks levels has been reported in many cereals, including rice (Ashikari et al. 2005), wheat (Ogonowska et al. 2019), maize (Hluska et al. 2016) and barley (Zalewski et al. 2014). There are 11 CKX homologues reported in the rice genome, 13 in maize, 7 in Arabidopsis, and wheat has 11 to 14 CKX gene family members (Chen et al. 2020). In rice, *Gn1a* encodes for the one of the *Cytokinin oxidase/dehydrogenase2* (*OsCKX2*). Loss of function or repression expression of the *OsCKX2* gene resulted in the accumulation of CKs in inflorescence meristems and promoted inflorescence meristem activity, thus producing a large panicle architecture with an improved grain number per panicle (Ashikari et al. 2005; Li et al. 2013; Yeh et al. 2015; Tu et al. 2022). Target mutagenesis was employed through gene editing in the *Gn1a* locus, resulting in increased plant height, panicle size, and number of flowers per panicle in the japonica rice cultivar Zhonghua 11 (Li et al. 2016). Furthermore, studies also reported that loss of function in CKs biosynthesis pathway genes resulted in a smaller panicle architecture with low a grain number (Gu et al. 2015; Kurakawa et al. 2007; Kuroha et al. 2009). Thus, the above studies indicate that endogenous cytokinin content in the reproductive tissue is crucial for the development of reproductive architecture.

CK biosynthesis and its signaling components are well connected in drought responses in plants. Stress stimuli as well as cellular signaling influence CK response and translocation, shaping physiological and molecular reactions to source/sink alterations, delayed senescence, and grain yield (Hai et al. 2020). Growing evidence suggests that CK mediates drought stress responses through crosstalk with ABA, auxin, SA, and brassinosteroids (Hai et al. 2020). Ghosh et al. (2018a, b) found that drought conditions decrease expression of the CK biosynthesis adenosine *phosphate-isopentenyl transferase* genes such as *AtIPT3* and *AtIPT9*. Research findings also indicate that increasing endogenous CK levels through overexpression of the CK biosynthesis gene IPT showed significant drought tolerance in a variety of crops, including creeping bentgrass (Merewitz et al. 2012; Xu et al. 2016; Xu and Huang 2017), rice (Peleg et al. 2011; Reguera et al. 2013), peanut (Qin et al. 2011), canola (Kant et al. 2015), cotton (Kuppu et al. 2013), eggplant (Xiao et al. 2017), tropical maize (Bedada et al. 2016) and wheat (Joshi et al. 2019). The cytokinin oxidase family members involved in the CK degradation mechanism also play an important role in various physiological and developmental modifications of plants under drought stress (Hai et al. 2020). It was clearly observed that in soybean, *GmCKX07* and *GmCKX13* showed higher transcriptional activity in roots and root hairs under low water availability (Le et al. 2012). A drought-induced increase in transcription activity in *BrCKX1-1*, *BrCKX1-2*, and *BrCKX5* had been observed during water limitation conditions (Liu et al. 2013). Based upon an expression analysis of foxtail millet (*Setaria italica*), it was found that, apart from *SiCKX2* and *SiCKX11*, other CKX transcripts were up-regulated under polyethylene glycol-induced drought stress and exogenous ABA application (Wang et al. 2014). Study results also showed that CK receptor histidine kinases, located mainly in the endoplasmic reticulum, were significantly up-regulated upon exposure to dehydration conditions in maize (Susan et al. 2013) and soybean (Le et al. 2011). Recent studies have revealed that CKs play both positive and negative roles in drought stress adaptation in plants (Zalabák et al. 2013). Many in planta studies have shown that CK has a negative regulatory role in drought stress response, with CK-deficient plants having a better capacity to survive and withstand drought stress (Pospíšilová et al. 2016; Vojta et al. 2016; Lubovská et al. 2014). Transgenic Arabidopsis and tobacco plants with overexpression of *AtCKX1* and *AtCKX3* exhibited increased root elongation, lateral root formation, enrichment of leaf mineral content, and improved drought tolerance (Werner et al. 2010). Drought tolerance was also shown in CK-deficient Arabidopsis mutants with the *ipt 1 3 5 7* genotype (Nishiyama et al. 2011). It was also found that AHP2/3/5 (Nishiyama et al. 2013), AHK3 (Kumar et al. 2014), and ARR1/10/12 (Nguyen et al. 2016) CK signaling component deficient mutants exhibit drought tolerance phenotypes. It appears that CKs play a significant role in drought response

mechanisms, and future functional studies should explore the tissue-specific and spatially inducible expression of CK metabolic genes in order to investigate the detailed role of these genes in drought tolerance in plants.

As the world population is expected to reach 9.2 billion by 2050, the existing conventional breeding approach will not be sufficient to meet global food demand. Advances in high-throughput genotyping and recently added genetic tools, including CRISPR/Cas9 genome editing, increase the ability to validate gene function, modify plant genomes in various ways, and address various bottlenecks related to productivity and other valuable traits. Rice is a staple food for almost half of humanity worldwide. Asia produces and consumes 80–90% of the world's rice, and indica rice accounts for three-quarters of the area of world rice production. *Gn1a* allele is common to many high-yielding indica rice varieties. MTU1010 is one of the most popular mega rice variety grown on a large scale in India. Given the above facts based on RNA interference studies, the *OsCKX2* genes could regulate agronomic traits and be useful to further improve the productivity of the modern variety. Here, we endeavored to investigate the agronomic characteristics and grain architecture of the MTU1010 indica rice background through CRISPR/Cas9-mediated disruption of the *OsCKX2* locus. Furthermore, we conducted additional research to assess the influence of the mutation in the *OsCKX2* gene on drought and salinity tolerance.

Materials and methods

Plant material and chemicals

The MTU1010 indica rice genotype was used for the functional validation of the *OsCKX2* gene. Chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich, St Louis, MO, USA) and Duchefa Biochemie (The Netherlands). Cloning, nucleic acid isolation, and purification kits were procured from Thermo Fisher Scientific (USA). WT (wild type) is referred to the untransformed rice line.

Insilco analysis of *OsCKX2*

The genomic, coding, and protein sequences of *OsCKX2* and family members were obtained from the Rice Annotation Project (https://rapdb.dna.affrc.go.jp/) and Rice Genome Annotation Project (http://rice.uga.edu/). The conserved domain was predicted by CDD (https://www.ncbi.nlm.nih.gov/cdd) and Pfam (http://pfam.xfam.org/). *OsCKX2* expression in different tissues was presented by (https://bar.utoronto.ca/eplant_rice/).

Vector designing and plasmid construction

The 20 nt specific guide RNA (gRNA) spacer sequence (5'-ACGTGCTAGAGCTCGACGTC-3') was selected at 320-340 bp downstream of the start codon (ATG) within the first exon of the cytokinin dehydrogenase domain (*LOC_Os01g10110*; *Os01g0197700*) based on the Insilco tool CRISPR-direct (https://crispr.dbcls.jp/) against the indica rice genome (Fig. S1). The 20 nt gRNA spacer sequences along with the 4 nt *BsaI* cloning sites were chemically synthesized. The gRNA oligo-duplex was developed by mixing forward and reverse primers and subsequently cloned into the *Bsa*I digested U6-sgRNA entry vector 1 (EV1-U6-sgRNA) following the protocol Achary and Reddy (2021). The *Cas9* expression cassette containing the rice codon-optimized enhanced *Streptococcus pyogenes Cas9* (*eSpCas9*) gene was prepared under the Zea mays ubiqitin1 promoter (ZmUbiP) and nopaline synthase terminator (NosT) in the subcloning entry vector (EV1). The EV1-(ZmUbiP-*eSpCas9*-nosT) was initially cloned into the pMDC99 plant transformation binary vector following the LR recombinase gateway cloning method, followed by blank entry vector 2 (EV2). The EV1-(OsU6-*CKX2*-gRNA-PolIIIT) was finally cloned into the resultant pMDC99 clone after a second LR recombination reaction following the method of Achary and Reddy (2021).

Genetic transformation and molecular identification of targeted mutagenesis in the transformants

The resultant recombinant pMDC99 vector (pMDC99 + U6-sgRNA + *eSpCas9*) was confirmed by sequencing and transferred into *Agrobacterium*-EHA105 cells. The putative T0 rice lines were developed following the *Agrobacterium*-mediated rice transformation protocol method as described by Achary and Reddy (2021). The ZmUbi promoter forward and *Cas9* gene reverse primers were used to confirm positive putative T0 rice lines (Table S1). To confirm insertion-deletion mutation (INDEL) among the T0 and their progeny rice lines, a pair of unique flanked primers were designed around spacer gRNA targets (Fig. S2; Table S1) of the *OsCKX2* genomic clone. The amplified products (774 bp) obtained from rice lines were initially gel-purified, and further, the eluted PCR products were directly sent for Sanger's sequencing (Macrogen, Korea) to confirm targeted mutagenesis in the *OsCKX2* locus.

Quantification of phytohormones

LC–MS was used for the quantification of phytohormone cytokinin (*trans*-zeatin) and IAA (indole acetic acid) following the method of Vadassery et al. (2012). Exact 250 mg

fresh tissue samples were harvested and snap frozen in liquid nitrogen, immediately processed (recommended), or stored at -80 °C until use. Samples were ground into fine powder using liquid nitrogen, and further processing was carried out under ice-cold conditions. Add 1 mL of ice-cold extraction buffer (MeOH:H_2O:HCOOH in a ratio of 15:4:0.1) with an internal standard (10 µl internal standard/ml) and transferred it to the 1.5 mL centrifuge tube. Vortex for 10 min and keep in ice for 1 min. Centrifuge the samples for 10 min at 12,000 g at 4 °C and transfer the supernatant into 2 mL micro-centrifuge tubes. Repeat the above stem by adding 500 µL of extraction solvent (without internal standard) to the pellet, and re-extracting, and mixed the supernatant into the above 2 ml micro-centrifuge tubes. Condition a C18 RP SPE column with 1 mL of MeOH and 1 mL of 0.1% HCOOH and load the supernatant onto the pre-conditioned C18 RP SPE column. Following, wash the column with 0.1% HCOOH and 5% MeOH twice. Finally, elute with 1 mL of ice-cold 0.1% HCOOH in acetonitrile. Evaporate the solvents to dryness using speedvac. The pellet obtained was resuspended in 100 µL of 5% methanol (without internal standard) and immediately processed for analysis. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 3 4.6 mm, 1.8 mm; Agilent). Formic acid (0.05%) in water and acetonitrile were employed as mobile phases.

Quantification of cytokinin dehydrogenase enzyme activity

The cytokinin dehydrogenase enzyme activity in the panicle tissue was performed by Tsago et al. (2020). Briefly, panicle tissue samples from *wt* and *Osckx2* mutant lines were ground into fine powder and mixed with 2 mL of protein extraction buffer (50 mM potassium acetate, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 0.5 mM DTT and a 5 µL protease inhibitor cocktail) and centrifuged at 14,000 rpm for 15 min at 4 °C. Into the supernatant, a 15 µL aqueous solution mixture containing 5% (v/v) polyethyleneimine, 0.5 mM phenylmethanesulfonylfluoride, and 0.5 mM Nα-tosyl-L-lysine chloromethyl ketone was added. Following centrifugation, the clear supernatant was used for the measurement of CKX2 activity. The 400 µL reaction mixture containing 50 mM Tris–Cl buffer pH 8.5, 0.5 mM dichlorophenolindophenol, and 0.15 mM N 6-isopentenyl adenine (iP) as substrate was mixed with 200 µL of the above supernatant protein. The mixture was initiated by placing it in a water bath at 37 °C for 1 h. Following incubation, the reaction was stopped by adding 300 µL of 40% trichloroacetic acid. The mixture was centrifuged at 14,000 rpm for 5 min at 4 °C. Further, 200 µL of 4-aminophenol (3%) prepared in 6% trichloroacetic acid was added to the above reaction tube and incubated at room temperature for 10 min. The formation of Schiff base was monitored at 352 nm by spectrophotometer (Ultrospec 7000 Dual Beam). The CKX activity was expressed as pkat mg^{-1} protein. Bradford method was used for the determination of protein content in the sample extract.

Drought assay

Rice seeds were surface sterilized in 0.5% Bavistin for 5 min, followed by thorough washing in sterile water for 5 min. To check drought tolerance, around 12 seeds were transferred into a 12 celled plastic seedling tray and germinated on vermiculite. The seedling trays were placed inside a square tray filled with nutrient Yoshida medium and grown for 21 days under greenhouse conditions of 70–80% relative humidity at ± 28 °C. The drought stress was imposed on 21-day-old plant for 10 days with holding water by removing excess Yoshida medium from the vermiculite and placing the seedling tray on a bench for 30 min to drain out excess nutrient medium. Following drought stress, the seeding tray was placed on Yoshida nutrient solution during the recovery (rehydration) phase. During the 7th day of the post-recovery period of drought stress treatment, survival percentage (%) was calculated. Similar setup seedling trays without drought treatment were maintained and handled under watering conditions as a control. Most of the wild-type (WT) control plants had reached a transitional stage of wilting by the end of the fifth day following drought; therefore, we chose the fifth day of drought to monitor the various physiological and biochemical responses. Before commencing the drought experiment, the initial uniformity of soil water status was maintained in both the wild-type and *Osckx2* mutant groups (WaterScout SMEC soil moisture meter, Spectrum Technologies, USA).

Measurement of transpiration

The water use efficiency of plants is closely related to leaf transpiration and its performance in drought-prone environments. Transpiration water loss was measured by various methods, including the excise leaf water loss assay, relative water content, and transpiration water evaporation. Water loss rates from detached leaves were estimated in accordance with Verslues et al. (2006). The rate of water loss from the leaf surface was calculated at different time intervals by air drying the detached leaves from the WT control and *Osckx2* mutants from the fully expanded penultimate top leaves of 2-month-old plants. Following detachment, the initial fresh weight (FW) was immediately recorded, followed by the turgid weight (TW), which was measured by soaking the leaf in water for 2 h. Dehydration stress was induced by air drying leaves over a bench at room temperature, and leaf weight was measured at

different time intervals (15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, and 300 min). Dry leaf weights (DW) were measured after 48 h of 80 °C oven drying. Water loss% was calculated by leaf weight after air exposure to its initial fresh weight of the tissue. The following formula was used to calculate water loss (WL) % = [(FW–DW)]/FWx100. In addition, the relative water content (RWC) was calculated using the following formula RWC (%) = [(FW-DW)/(TW-DW)]X100. Thirty-day-old WTs and *Osckx2* mutants were used to monitor transpiration water evaporation in glass bottles containing Yoshida solution for greenhouse conditions (Fig. 4). Before starting the experiment, bottles were carefully enclosed with cellular plastic foam to avoid any natural water evaporation. The transpiration water loss was calculated by the difference in the initial weight of the bottle with plants at 6 a.m. and final weight at 6 p.m. Water loss was normalized with leaf area and expressed in percentage per unit area.

Measurement of photosynthesis and photochemical efficiency

Photosynthesis parameters such as transpiration rate (*E*), photosynthesis rate (*A*), stomatal conductance (*gs*), and intercellular CO_2 concentration (*Ci*) were monitored on the fully expanded 2nd most top leaves of WT and *Osckx2* from five different plants using LICOR 6400 (LICOR, USA). The light intensity was set at 1000 mol m^{-2} s^{-1} photosynthetically active radiation (PAR), the flow rate was set at 400 mol s^{-1}, and the reference CO_2 concentration was maintained at 400 ppm. Data were collected from three technical experiments. Measurements were conducted between 10 a.m. and 12 p.m. on sunny days.

Infrared image-based phenotyping of canopy temperature

Infrared thermal camera (FLIR Systems Inc, USA) was used to measure plant canopy temperature. Trays containing 21-day-old WT and *Osckx2* plants were subjected to dehydration stress as mentioned above ("Materials and Methods" 2.7). During midday (1.00–2.30 PM), at a maximum ambient temperature of 32 °C, infrared images were taken before and 5 days after the drought stress treatment. Infrared thermal cameras have a total pixel resolution of 640 × 480 pixels and a wavelength band of 7–14 µm. Thermal images were used to measure the average surface temperature using FLIR SmartView 4.3 software. The data were collected from three technical experiments by selecting about 30-pixel points from each group.

Measurement of ROS, antioxidative enzyme and photosynthetic pigments

The lipid peroxidation of malondialdehyde (MDA) produced as a result of peroxidation of lipid membrane damage was measured spectrophotometrically by the thiobarbituric acid (TBA) assay described by Achary et al. (2012). Antioxidative enzymes including catalase, superoxide dismutase, ascorbate peroxidase, and nuclease activity were spectrophotometrically measured according to Achary et al. (2012). Chlorophyll content was estimated using dimethyl sulfoxide (DMSO) as described by Hiscox and Israelstam (1979), and electrolytic leakage was estimated using the Horiba LAQUAtwin EC-33 Compact Conductivity Meter. Proline content was measured by Bates et al. (1973).

Salinity tolerance assay

Rice seeds were surface sterilized in 0.5% Bavistin for 5 min, followed by thorough washing in sterile water for 5 min. WT and *Osckx2* mutant lines were allowed to grow in a seedling tray filled with yoshida solution for 21 days under the plant growth cabinet (Percival USA) with 70–80% relative humidity at ± 28 °C. The 21-day old plants were subjected to salinity stress for a duration of 15 days using varying concentrations of NaCl (ranging from 100 to 200 mM) in the Yoshida nutrient medium. Seedling trays without salinity treatment were maintained and handled in a similar way and were served as a control (Fig. S6). At the end of the salt treatment, root and shoot length, as well as survival rate, were monitored.

Field agronomy

Agronomy parameters such as plant height (PH), tiller number per plant (TNP), panicle length (PL), number of primary branches in the main panicle (PB), number of secondary branches in the main panicle (SB), grains in the main panicle (GnP), grain length (GL), grain width (GW), 1000-grain weight (TGW), and total yield per plant (TPY) were recorded at the plant maturity stage. The agronomy was performed at ICGEB, New Delhi, India (latitude 28° 31' N, longitude 77° 10' E) under containment natural field conditions covered by stainless steel during the months of June to October (temperature max 33–40 °C and min 25–28 °C).

Statistical analysis

Physiology and biochemical experiments were conducted in three separate technical replications using multiple biological samples and repeated twice or thrice. The mean values ± standard deviations (STD) are presented in the respective figures and tables. From the pooled data,

multiple comparisons were carried out with one-way and two-way analysis of variance (ANOVA) based on Tukey's test and least significance difference (LSD) at $p \leq 0.05$ and/or 0.01 levels. GraphPad Prism V8 was used to visualize statistical data. The data increase significantly at $p \leq 0.05$ (a) or 0.01 (b) and decrease significantly at $p \leq 0.05$ (c) or 0.01 (d) compared to WT under control and drought conditions. Similarly, the mean value showed a significant increase or decrease at $p \leq 0.05$ denoted (*) or 0.01 (**) in the same plant group (WT or *CKX2*-KO) under both control and drought conditions.

Results

Molecular characterization of genome-edited *Osckx2* rice lines

The expression plot developed using ePlant Rice (University of Toronto BAR browser; http://bar.utoronto.ca/) indicates that the *OsCKX2* gene is highly expressed in the panicle tissue, young developing seed, and shoot apical meristem tissue (Fig. 1a). We designed a specific spacer gRNA located at the first exon of the *OsCKX2* gene in the coding region of the dehydrogenase protein domain (Fig. 1b). We used the Zea mays ubiquitin1 gene promoter for strong expression of the *eSpCas9* gene. The rice U6

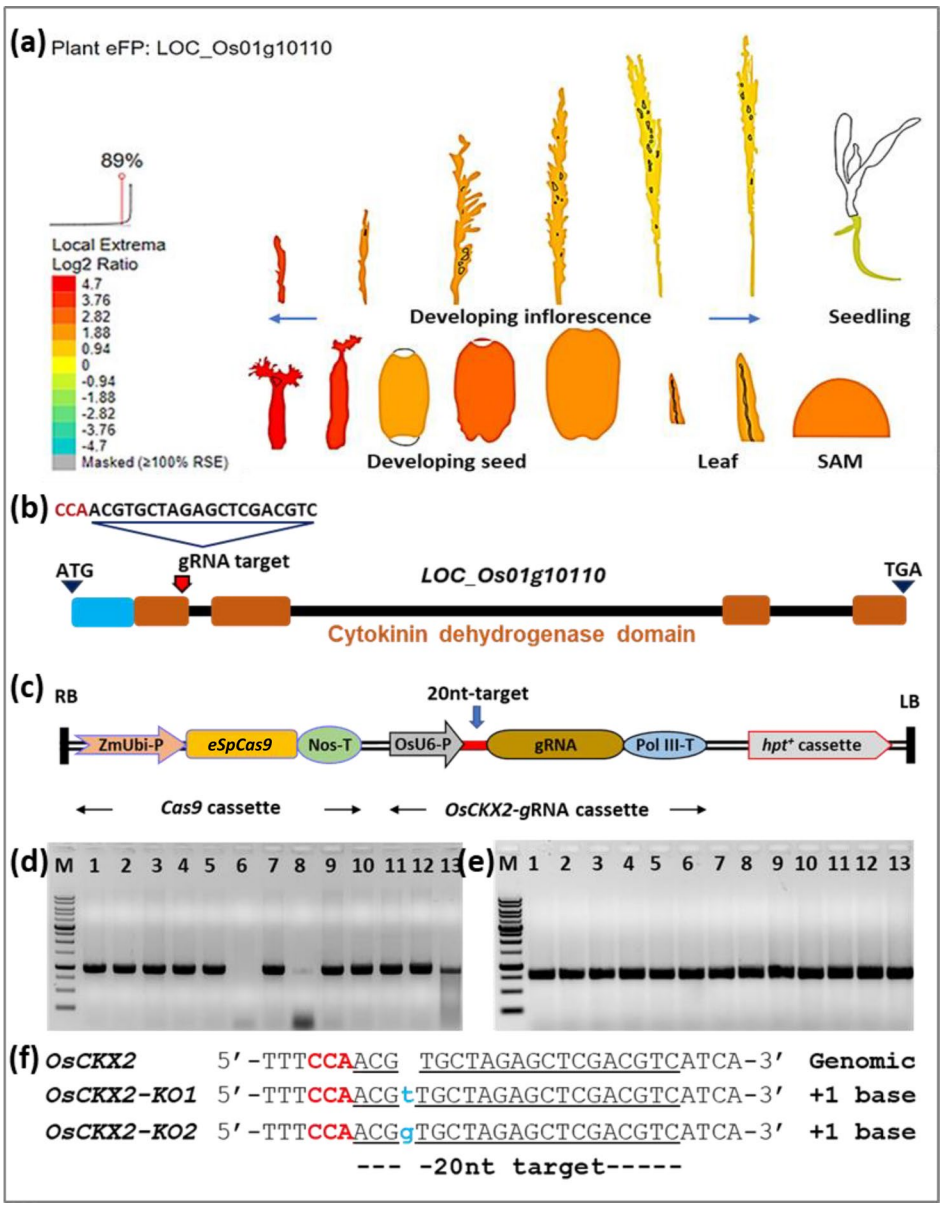

Fig. 1 *OsCKX2* structural organization, *OsCKX2* editing vector construct, and mutation analysis. **a** Tissue-specific expression of *OsCKX2* in rice panicle. **b** The structural organization of *OsCKX2* consists of 4 exons interrupted by 3 introns and the location of the gRNA target within the first exon of the cytokinin dehydrogenase domain. **c** T-DNA border showing genome-editing expression cassettes (*eSpCas9* and sgRNA) in the pMDC99 vector. **d** PCR confirmation of T0 rice lines showing absence or presence of *Cas9* (887 bp) genes. **e** PCR amplification of the 774 bp flanked product, including the sgRNA targets of the *OsCKX2* gene from the T0 plants. **f** Genotype confirmation of rice lines showing T-insertion (*Osckx2-1*) and G-insertion (*Osckx2-2*) mutations in the *OsCKX2* locus

promoter was used to express sgRNA. The final genome-editing vector tool (pMDC99-U6-sgRNA-*eSpCas9*) was transformed into the indica rice MTU1010 cultivar (Fig. 1c). Following tissue culture, 13 putative rice lines were generated. Eleven T0 rice lines showed PCR positive for the *eSpCas9* gene (Fig. 1d). PCR was performed to amplify the 774 bp flanked product, including the sgRNA targets of the *OsCKX2* gene, and sanger sequenced using a nested forward *OsCKX2* gene-specific primer from all the *eSPCas9*-positive plants (Fig. 1e, Table S1). We identified only a biallelic mutation with single nucleotide T-insertion among the T0 rice lines; subsequently, we referred to it as *CKX2*-KO1 or *Osckx2-1* (Fig. 1f, Fig. S3). We genotyped a total of 214 individual progenies from the T1 generation. Out of 214, we found a single G-insertion biallelic mutation named *CKX2*-KO2 or *Osckx2-2* (Fig. 1f, Fig. S3). Apart from the above mutation types, we did not notice any additional new INDEL mutations in the T1 progeny. The mutation effect on the protein frame was bioinformatically analyzed. Both of the above mutations end up with truncated *OsCKX2* peptides (Figs. S4 and S5). The *CKX2-KO1* and *CKX2-KO2* mutations were stabilized in the T2 and T3 generations. The T-DNA free *CKX2-KO* mutants were identified and further carried out for agronomy and stress physiology analysis.

Loss of function *OsCKX2* increases cytokinin levels in the inflorescence and leaf tissue by decreasing cytokinin dehydrogenase enzyme activity

The phytohormone trans-Zeatin (tZ) is highly abundant in the plant. Both the *Osckx2* mutants resulted in a significant ($p \leq 0.01$) increase in the levels of endogenous cytokinin (tZ) content in the inflorescence tissue, as confirmed by LC–MS/MS (Fig. 2a). The *Osckx2* mutants increased 114–128% tZ content compared to *wt*. Similarly, we have also observed increased cytokinin content in the flag leaf of *Osckx2* mutant lines. Compared to panicle tissue, the flag leaf contains more tz content both in the WT and *Osckx2* mutant lines (Fig. 2a). It is interesting to note that the loss of *OsCKX2* function led to a significant increase in the auxin IAA (indole acetic acid) content in the flag leaf (Fig. S7). We studied the effect of mutation on the cytokinin dehydrogenase enzyme activity in the panicle tissue. Compared to the WT plant, the CKX enzyme activity level in the panicle tissue was significantly ($p \leq 0.01$) reduced by 32–37% (Fig. 2b). From the study, we confirmed that the CKX2 enzyme is significantly active in the panicle tissue and leaf tissues. Loss of function of *OsCKX2* loci resulted in remarkably increased cytokinin content in the panicles of *Osckx2* rice lines.

ckx2 mutants alter vegetative morphology, improve panicle morphology and grain yield in rice

The agronomic performance of *CKX2*-KO mutants was recorded under field and greenhouse conditions (Figs. 2c-k). The *CKX2*-KO plant showed slightly reduced plant growth morphology without a significant change in tiller number (Fig. 2c, d and k; Table 1). The flag leaf length (25–28%) and flag leaf area (25–27%) were significantly ($p \leq 0.01$) increased compared to WT (Fig. 2e; Table 1). Furthermore, we studied the effects of the *CKX2* mutation on the reproductive architecture. *CKX2*-KO mutants significantly improve panicle primary branch (19–21%), secondary branches (22–23%), panicle length (6–8%) and number of seeds in the main panicle (40–42%) compared to WT panicle (Fig. 2f; Table 1). Before the grain filling stage, the hull size of *CKX2*-KO lines slightly improved, this was reflected in the increased 1000 husk weight of *CKX2*-KO rice lines. The improved hull size leads to improved grain architecture in the *CKX2* mutant (Fig. 2g–j; Table 1). We reported 19–21% increased width and 19–22% enhancement in seed length in *CKX2*-KO lines (Table 1). Similarly, the *CKX2*-KO lines significantly improved the 1000-grain weight by 5% (Table 1). Further, the grain yield in the *Osckx2* mutant per plant was significantly improved ($p \leq 0.01$), up to 16–23% more compared to the WT (Table 1). Further study indicates that the increased grain yield in the *Osckx2* mutant was due to the great contribution of increased secondary branches in the main panicle (Fig. 2f; Table 1). Our result indicates that the functionally active *CKX2* allele negatively affects grain width, length, secondary branch, and grain yield in rice. However, we did not notice any change in the tiller number in the *Osckx2* mutant (Table 1). From the above finding, we conclude that the *OsCKX2* gene expression greatly influences flag leaf and reproductive architecture, including panicle, seed length, seed weight, and number of secondary branching in rice. Further, we assume that the plant hormones auxin and cytokinin and their particular endogenous balance shape the plant morphology. Therefore, alteration of the auxin-cytokinin balance greatly influences plant morphology.

ckx2 mutants showed improved survival rate during drought stress

Growth and survival performance of *Osckx2* under drought were assessed by withholding irrigation conditions (Fig. 3). The dehydration tolerance of the plants was determined by monitoring leaf turgidity, leaf rolling, and survival rate following the seventh day after the post-stress recovery period. By the end of the third day after withholding water, both *Osckx2* and WT almost exhibited similar phenotypic characteristics, but WT displayed slight leaf rolling drought

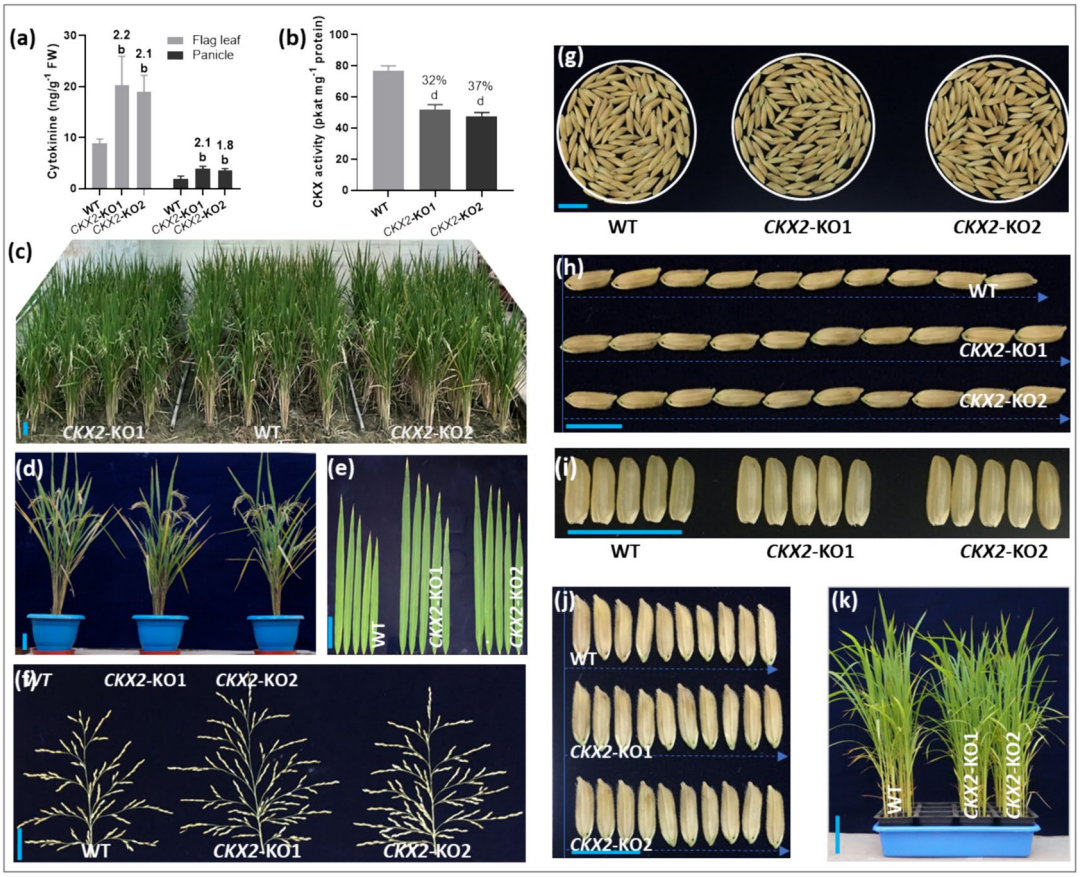

Fig. 2 Cytokinin content and field agronomy of WT and *Osckx2* mutants. **a** The LC–MS quantification of cytokinin content in flag leaf and panicle tissue. The cytokinin content was substantially increased ($p \leq 0.01$; **b**) in *Osckx2* mutants compared to WT plants. Data mean ± SD ($n = 3$). **b** The cytokinin dehydrogenase enzyme activity in the panicle tissue was significantly decreased ($p \leq 0.01$; **d**) in *Osckx2* mutants compared to WT. Data mean ± SD ($n = 9$). The WT and *Osckx2* rice lines were grown under natural containment (**c**) field net-houses (**d**) and greenhouse conditions. The *Osckx2* lines showed improved (**e**) flag leaf and (**f**) main panicle length compared to the WT plant. The grain phenotype of *Osckx2* exhibited increased (**g**) 100 seed volume, (**h**) seed length, (**i**) grain width and (**j**) seed width corresponding to WT plant. **k** Plant morphology of 3-week-old WT and *Osckx2* mutants. Agronomy data, mean ± SD ($n = 15$). Significant differences between the groups (WT and CKX2-KO) increase at $p \leq 0.01$ (**b**) or decrease by 0.01 (**d**). The scale bar for all figures except **c**, **d**, **e**, **f** and **k** (5 cm) measures 1 cm

Table 1 Field agronomic performance of WT and *Osckx2* lines

Plant groups	Plant height (cm)	No. of tiller/plant	Main flag leaf Length (cm)	Flag leaf Area (cm^2)	Main panicle length (cm)	MPPB	MPSB
WT	109.8 ± 5.5	9.3 ± 1.5	32.9 ± 4.4	31.3 ± 5.4	25.2 ± 0.8	10.4 ± 0.5	35.1 ± 1.1
CKX2-KO1	102.6 ± 4 (NS)	9.7 ± 1.7 (NS)	42.4 ± 3.9 (b)	41.8 ± 5.7 (b)	26.9 ± 0.7 (b)	12.4 ± 0.5 (b)	43.4 ± 1.3 (b)
CKX2-KO2	101.1 ± 4.4 (NS)	9.5 ± 1.8 (NS)	41 ± 2.6 (b)	40 ± 2.8 (b)	27.2 ± 0.6 (b)	12.6 ± 0.4 (b)	43.1 ± 1.2 (b)
Plant groups	No of grains/ main panicle	Grain length (cm)	Grain width (mm)	1000 grain weight (g)	1000 hull weight (g)	Total grains/ plant	Grain yield/plant (g)
WT	158.67 ± 11.3	0.77 ± 0.04	2.26 ± 0.1	16.7 ± 0.26	4.1 ± 0.12	1137.5 ± 52	22.5 ± 1
CKX2-KO1	225.4 ± 12.8 (b)	0.94 ± 0.05 (b)	2.74 ± 0.1 (b)	17.6 ± 0.24 (b)	4.5 ± 0.22 (b)	1305.3 ± 74 (b)	27.7 ± 1.1 (b)
CKX2-KO2	222.8 ± 12.7 (b)	0.92 ± 0.05 (b)	2.7 ± 0.1 (b)	17.5 ± 0.23 (b)	4.3 ± 0.26 (b)	1255.6 ± 92 (b)	26.1 ± 1.6 (b)

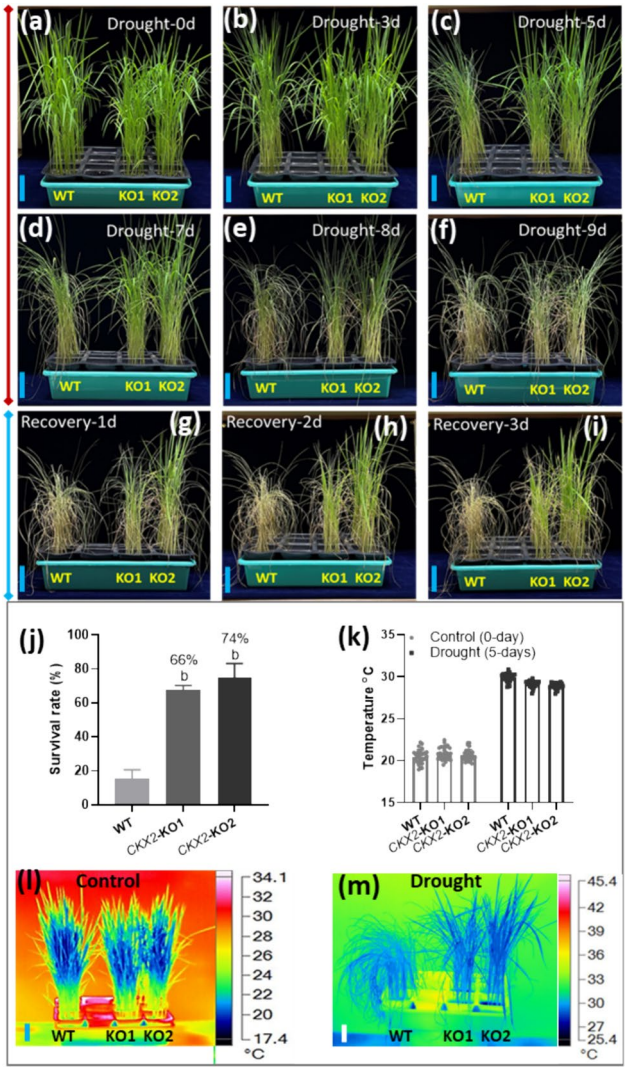

Fig. 3 Response of *WT* and *Osckx2* mutants to water-limiting condition. **a**–**f** Drought stress was applied to 20-day-old WT and *Osckx2* seedlings with holding water in a greenhouse for 9 days, followed by (**g**–**i**) recovery with yoshida solution. **j** The *Osckx2* mutant showed an improved survival rate during the recovery period from drought compared to the WT group. Data ($n = 144$) mean ± SD. **k**-**m** Thermal imaging of WT and *Osckx2* seedlings over normal and stimulated drought (5th day) conditions. The *Osckx2* mutants displayed a decrease in leaf temperature following a 5-day drought treatment compared to WT. Data from three trays (108 ± SD). Significant differences between the groups (WT and *CKX2*-KO) increase with $p \leq 0.01$ (**b**). The scale bar denotes 5 cm

symptoms. WT leaves showed clear signs of leaf rolling and wilting on the day 5th after drought treatment (Fig. 3c). In contrast, the *Osckx2* rice seedlings start rolling around on day 5th of the drought and the clear rolling and wilting symptoms appear on the 8th day (Fig. 3e). After 10 days of drought stress, most of the leaves of WT and *Osckx2* seedlings had completely wilted, collapsed, and dried out, but a few leaves of *Osckx2* retained improved turgidity and appeared slightly greener than WT. During the recovery phase after 10th days of drought, *Osckx2* seedlings began to reopen their leaves after 12 h of rehydration and almost fully opened after 24 h (Fig. 3g). In WT, similar observations were delayed, as only a few plants had half-dried and half-opened leaves after 48 h of recovery (Fig. 3h). Moreover, post-recovery growth of WT was much slower than *Osckx2*. The *Osckx2* recovered rapidly and showed improved growth (Fig. 3i). Contrast to WT seedlings, which had a survival rate of 15%, mutant *Osckx2* plants showed a survival rate of 66–74% (Fig. 3j). The findings revealed that *OsCKX2* negatively impacts drought tolerance, and its inactivation enhanced survival rate under dehydration conditions.

Improved stress tolerance for *Osckx2* is positively correlated with canopy temperature reduction

As high temperature increases cellular damage, canopy cooling is crucial for plant fitness. Under water-deficit conditions, canopy cooling must be balanced with excessive water loss. Water evaporation is an endothermic process that absorbs heat from the canopy surface, thereby keeping the canopy cooler than the ambient temperature. Under normal growth condition, both WT and *Osckx2* mutants showed similar canopy temperatures (Fig. 3k-m). *Osckx2*, on the other hand, displayed a cooler canopy temperature under water-limiting conditions compared to WT plants (Fig. 3k-m). This could be due to the fact that the *Osckx2* mutant may maintain reduced transpiration and enhanced water conservation capacity compared to WT rice. During extreme drought conditions, *Osckx2* mutants were shown to demonstrate an improved survival rate due to evaporative transpiration cooling, which decreased the risk of cellular damage.

Osckx2 mutant transpire less water and exhibit improved drought tolerance due to increased cellular hydration

Soil water availability determines the intensity of drought stress, and a plant's drought tolerance varies depending on leaf water status, stomatal transpiration, and soil moisture content. Transpiration is the process through which plants shed more than 90 percent of the water they absorb. Measuring the relative water content (RWC) in leaves has been considered a reliable parameter for determining plant water stress status. The excise leaf water loss assay clearly demonstrated that the *Osckx2* mutant transpires less water than the WT plant (Fig. 4a-b). There is a significant difference in the percentage of water loss, and RWC was observed after the 90 min onset of dehydration stress. Compared to the WT control leaf, the *Osckx2* mutant appeared to have relatively higher RWC and reduced water loss (Fig. 4a-d). This finding

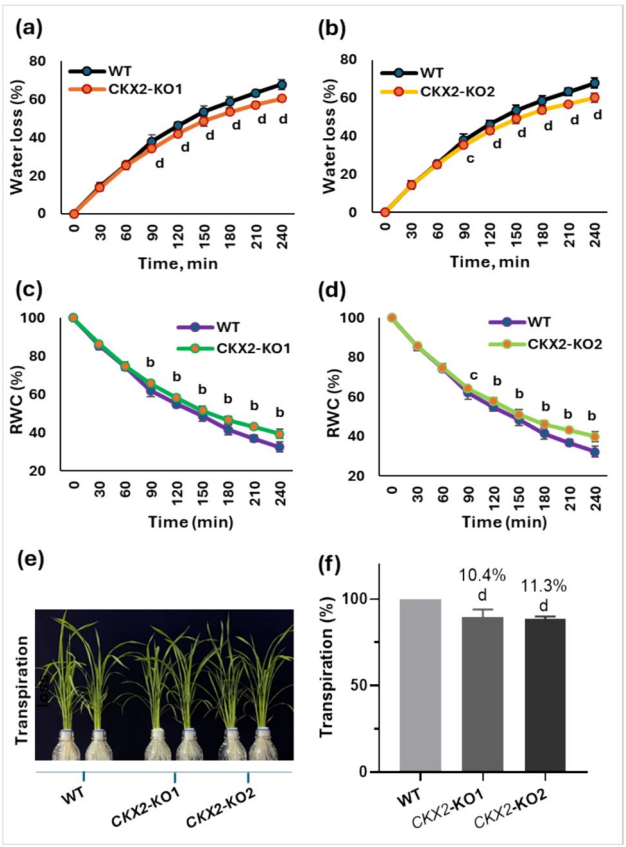

Fig. 4 Response of *WT* and *Osckx2* mutants to transpiration evaporation. **a**–**d** The detached leaf assay indicated that the *Osckx2* mutant exhibited a significantly ($p \leq 0.05$ (**c**) 0.01; **d**) lower percentage of leaf water loss and a higher relative water content (RWC) than the wild type (WT) leaf. Data mean ± SD (n = 9). **e–f** The whole plant transpiration water loss suggests that the *Osckx2* mutant exhibited a 10–11% reduction in leaf evaporation compared to the WT plant. Data mean ± SD ($n = 9$). Significant differences between the groups (WT and *CKX2*-KO) decrease at $p \leq 0.05$ (**c**) and 0.01 (**d**); increase $p \leq 0.01$ (**b**). The scale bar denotes 5 cm

conclusively indicates that *Osckx2* mutants lose less water during transpiration, thereby improving cellular leaf turgidity during drought.

In further studies, we investigated whether the improved drought tolerance of *Osckx2* could be attributed to more soil water availability and reduced transpiration evaporation, which delays symptoms of dehydration. The hydroponic leaf transpiration experiment clearly confirmed that *Osckx2* mutant lines transpired substantially less water than WT plants (Fig. 4e-f). Compared to WT plants, the *Osckx2* rice lines showed significantly reduced whole-day transpiration water loss by 10–11%. Our results suggest that *Osckx2* evaporates less water through leaf transpiration and significantly conserves more soil water than WT control plants. Consequently, *Osckx2* plants, which prevent dehydration stress, are able to maintain adequate leaf moisture levels for physiological and biochemical functioning. The fact that only a small number of WT plants survived in the survival study also backs up the current conclusion that WT plants lost more water through transpiration, which quickly depleted soil water and led to low leaf water potential, which explains why WT plants died quickly under prolonged drought conditions (Fig. 3a-j).

Osckx2 enhances photosynthetic performance under dehydration conditions

We investigated whether decreasing transpiration affected CO_2 uptake, carbon assimilation, and photosynthesis efficiency in *Osckx2*. To test the above objective, WT and *Osckx2* rice lines were grown up to 21 days old and subjected to drought stress under holding water conditions. Under normal water availability conditions, intercellular CO_2 concentration (Ci) and transpiration rate (E) were significantly decreased compared to WT, except stomatal conductance (*gs*), which remained unchanged (Fig. 5). On the other hand, the photosynthesis rate (*A*), water use efficiency (WUE), and photosynthesis capacity (Pn/Ci) were increased in *Osckx2* rice lines (Fig. 5). Except for *Ci*, drought stress significantly decreased all the above photosynthetic parameters in both WT and *Osckx2* rice lines. Furthermore, except for *E* and *gs*, the other photosynthetic parameters such as *A*, *WUE*, and *Pn/Ci* were significantly higher in the *Osckx2* lines compared to the WT plants on day 5th limited water conditions (Fig. 5). The result indicates that *Osckx2* rice maintained improved photochemical efficiency under drought conditions. The improved *WUE* of the *Osckx2* mutant resulted in enhanced photochemical performance and increased carbon assimilation under drought conditions.

Osckx2 mutant activates antioxidant machinery and osmo-protectants under water stress to protect cellular components and photosynthetic apparatus

Reactive species-mediated cellular damage is one of the common physiological consequences of plants experiencing environmental stresses. Oxidative bioindicators, such as electrolytic leakage and lipid peroxidation, are sensitive parameters of cellular injury. Even though the level of electrolytic leakage and lipid peroxidation (MDA) increased ($p \leq 0.01$) in both *Osckx2* and WT under drought stress, conversely, the above oxidative bioindicators were substantially less ($p \leq 0.01$) found in *Osckx2* liens with respect to the WT plant (Fig. 6a-b). In addition, we quantified major antioxidative enzyme status to confirm the hypothesis that improved drought tolerance in *Osckx2* might be due to improved antioxidative enzymes. Except for SOD, the CAT and APX enzymes were significantly increased in the *Osckx2* mutant under normal condition (Fig. 6c-e).

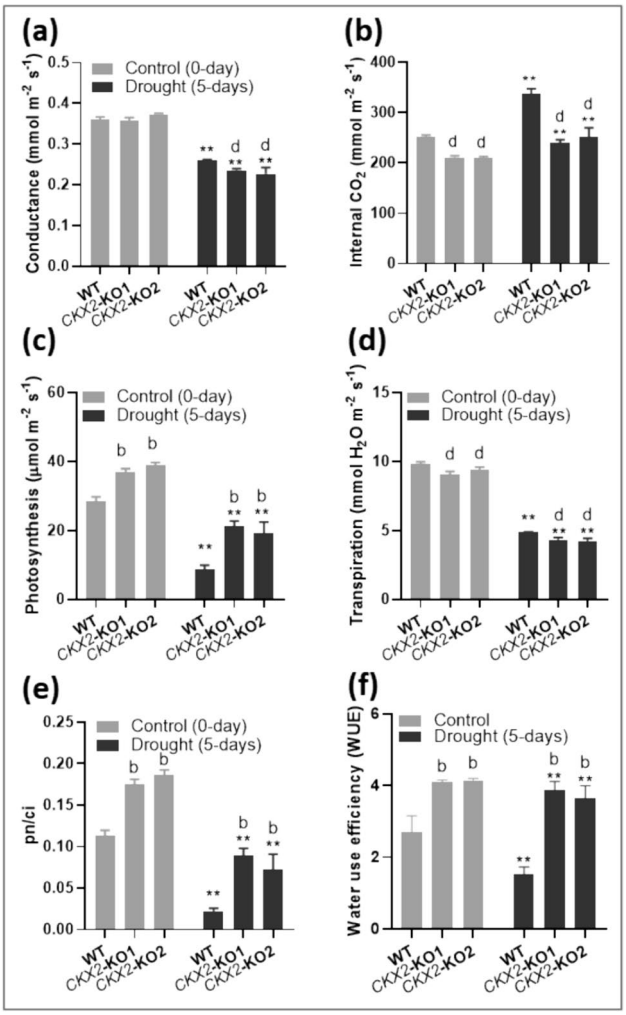

Fig. 5 Effect of photosynthesis on WT and *Osckx2* under control and drought conditions. Drought stress was imposed on 3-week-old WT and *Osckx2* seedlings by withholding water, and photosynthetic parameters were assessed after 5 days of drought. Drought stress significantly reduced (**a**) conductance (*gs*), (**b**) intercellular CO_2 concentration (*Ci*), and (**d**) transpiration rate (*E*) in *Osckx2* mutants compared to WT plants. In addition, *Osckx2* demonstrated improved (**c**) photosynthetic rate (*A*), (**e**) photosynthesis capacity (*Pn*/*Ci*), and (**f**) water use efficiency (*WUE*). Data mean ± SD ($n = 15$). Significant differences ($p < 0.05$ or 0.01) in means are indicated by letters and symbols. Significant differences between the groups (WT and *CKX2*-KO) increase with $p \leq 0.01$ (**b**) or decrease by 0.01 (**d**), and within-group increase or decrease at $p \leq 0.01$ (**)

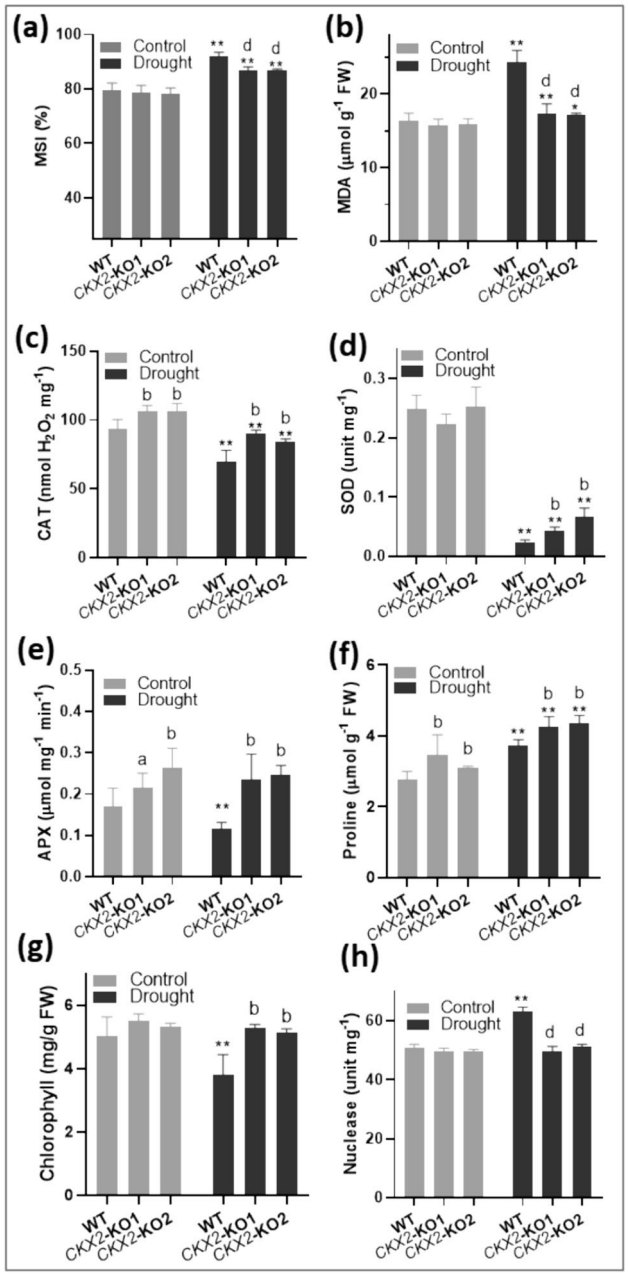

Fig. 6 Biochemical functioning of WT and *Osckx2* plants to drought condition. Under drought circumstances, (**a**) MSI% and (**b**) lipid peroxidation levels were much lower in the *Osckx2* mutant relative to the WT. The *Osckx2* rice lines exhibited notable enhancements in (**c**) catalase, (**d**) superoxide dismutase, and (**e**) ascorbate peroxidase activity, as well as increased (**f**) proline content and (**g**) chlorophyll content, except for reduced (**h**) nuclease activity under 5th day of dehydration conditions compared to WT. Data mean ± SD (n = 9). Significant differences ($p < 0.05$ or 0.01) in means are indicated by letters and symbols. Significant differences between the groups (WT and *CKX2*-KO) increase at $p \leq 0.05$ (**a**) and 0.01 (**b**) or decrease at 0.01 (**d**), and within-group increase or decrease at $p \leq 0.05$ (*) and 0.01 (**)

Drought stress significantly decreased CAT and SOD in both WT and *Osckx2* mutants. However, in the case of APX, the *Osckx2* mutant lines remain unchanged both under normal and drought conditions, but the APX level was significantly decreased ($p \leq 0.01$) in WT plant (Fig. 6e). Overall, *Osckx2* showed higher ($p \leq 0.01$) levels of CAT, APX, and SOD antioxidant enzyme activity under drought conditions than WT plants. The osmoprotectant proline is generally produced under stress conditions. In both WT and

Osckx2 mutants, drought stress increased proline content, but *Osckx2* mutants accumulated significantly ($p \leq 0.05$ or 0.01) higher proline content than WT (Fig. 6f). Photosynthetic pigments and the apparatus are negatively affected by water stress, which reduces the degree of photosynthesis and plant performance. Under normal conditions, total chlorophyll contents in WT and *Osckx2* mutants remained unchanged (Fig. 6g). In comparison to WT, the chlorophyll content was significantly ($p \leq 0.01$) greater in *Osckx2* under drought conditions (Fig. 6g). Plants show apoptosis-like cell death under stress triggered by PCD-associated endonucleases that cleave cellular DNA (Reape and McCabe 2008). We quantified the level of nuclease activity that is elevated during drought stress-induced senescence conditions. The level of nuclease activity in *Osckx2* is significantly lower in drought compared to WT (Fig. 6h). The findings from this study provide insight into the role of *Osckx2* mutants in adapting to drought stress conditions through the regulation of ROS homeostasis mechanisms. Based on the findings of this study, it appears that the *Osckx2* mutants are able to effectively protect essential biomolecules under drought conditions through an efficient oxidative protective mechanism. The improved photosynthetic performance of *Osckx2* under drought conditions could be directly associated with intact photosynthesis machinery, and this may explain why *Osckx2* lines recovered quickly from prolonged drought conditions (Fig. 2). It is possible that higher water status in *Osckx2* rice lines may protect the photosynthesis pigments and enhance photosynthetic performance during droughts (Fig. 5).

Osckx2 mutants improved osmotic tolerance under mannitol stress

To determine the function of *OsCKX2* under osmotic stress, we examined the response of *Osckx2* knockout lines to mannitol solutions of varying concentrations. WT seedlings were more sensitive to osmotic stress than the *Osckx2* line. Under 6 days of mannitol stress, wilting started on both WT and *Osckx2*; however, *Osckx2* revived quickly after the osmotic stress was removed during the recovery period (Fig. 7a-d). During the recovery phase, the WT plants revived slowly and most of them lost their viability, showing only a 56% survival rate (Fig. 7e). On the other hand, *Osckx2* mutant plants recovered quickly and showed improved survival (72%) compared to WT (Fig. 7e). Similarly, on day 7th of the recovery phase, fresh weight was significantly higher in *Osckx2* compared to the WT plant (Fig. 7f). These results suggest that WT seedlings are more sensitive to osmotic stress than *Osckx2* mutants.

Loss of *OsCKX2* gene function has no impact on salinity stress

To investigate the role of *OsCKX2* in response to salinity stress, *Osckx2* knockout lines were treated with different levels of NaCl solutions. WT seedlings and *Osckx2* lines exhibited similar responses. After 14 days of exposure to a 100 mM salt treatment, both WT and *Osckx2* plants displayed symptoms of wilting and leaf death. The intensity was higher in the 150 mM salinity treatment (Fig. S6). In addition, both WT and *Osckx2* seem to be dead after the 200 mM NaCl treatment. In addition, we did not notice any significant root and shoot growth improvement between the WT and *Osckx2* groups (Fig. S6). Both the root and shoot length showed a significant decrease in both WT and *Osckx2* as the concentration of NaCl solution increased in comparison to the control group (Fig. S6). These findings indicate that the loss of *OsCKX2* gene function did not impact salinity, and mutant *Osckx2* seedlings responded similarly to WT seedlings under salinity stress.

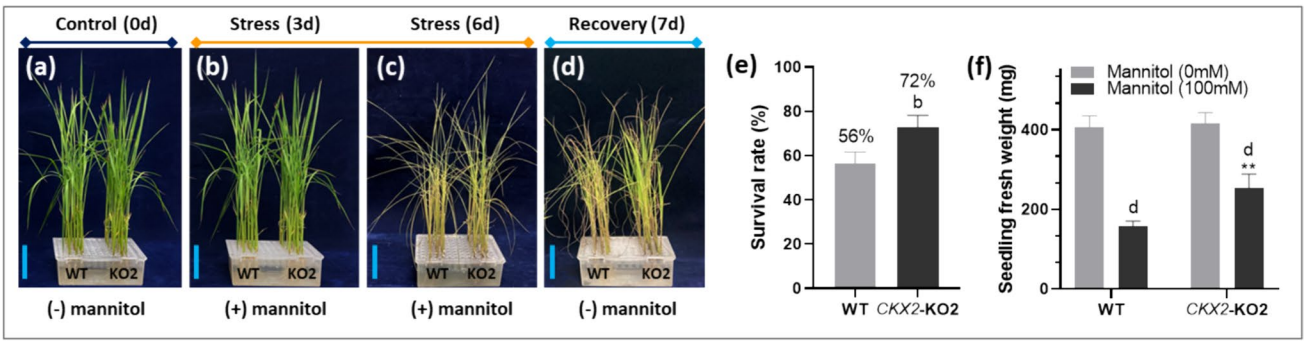

Fig. 7 Effects of mannitol stress on seedling growth and survival. **a**–**d** Three-week-old WT and *Osckx2* seedlings were subjected to osmolyte mannitol (100 mM) treatment under the plant growth chamber for 6 days, followed by the recovery of seedlings with yoshida solution. In comparison to the WT group, the *Osckx2* mutant exhibited enhanced (**e**) survival rate and (**f**) seedling fresh weight during the recovery period. Data ($n = 30$) mean ± SD. Significant differences ($p < 0.05$ or 0.01) in means are indicated by letters and symbols. Significant differences between the groups (WT and *CKX2*-KO) increase at $p \leq 0.01$ (**b**) or decrease by 0.01 (**d**), and within-group increase or decrease at $p \leq 0.01$ (**). The scale bar denotes 5 cm

Loss of *OsCKX2* function leads to increased ABA in rice seedling

Abscisic acid plays a vital role in stress-related responses, is involved in various plant development processes, and regulates numerous physiological and biochemical processes that enable plants to adjust to dehydration conditions. The LC–MS quantification data confirmed that the *OsCKX2* deletion mutation increased endogenous ABA under normal growth condition. Compared to the WT plant, the leaf ABA accumulation was substantially higher in *Osckx2* mutants (Fig. 8). In order to gain a deeper understanding of the role of *Osckx2* ABA-responsive pathways in rice, further investigation is required.

Discussion

Improving the genetic potential of cereals is crucial to food security. Panicle architecture is one of the key components of grain yield. Identification of genes and regulatory mechanisms that control inflorescence morphology is important for the development of high-yielding rice varieties (Xing and Zhang 2010). Genetic variations in such key regulatory genes are of paramount importance in crop improvement program. Natural variations associated with important agronomic traits are widespread in the genomic background of different cultivars, thus limiting their immediate use to improve existing cultivars. CRISPR/Cas9 genome editing has been used as part of modern breeding technology to create desired targeted mutations in several crops. Here, we successfully utilized the CRISPR/Cas9 method and developed knockout mutants of the *OsCKX2* gene in the high-yielding MTU1010 indica cultivar. This finding will facilitate the pyramiding of this useful resource, either for breeding purposes or for the analysis of gene regulatory networks. The *OsCKX2* gene is synonymous with *Gn1a* (*grain number 1a*) which negatively controls panicle branching pattern and grain number (Ashikari et al. 2005; Yeh et al. 2015; Tu et al. 2022). Cytokinin oxidases are the only enzymes that catalyze the breakdown of many active forms of various cytokinins in plants. Therefore, we predicted that there would be an alteration in the level of endogenous cytokinin in the *Osckx2* mutant. As previously reported, our study also showed that loss of function of *OsCKX2* resulted in the accumulation of higher levels of cytokinin in both panicle and leaf tissues (Ashikari et al. 2005; Joshi et al. 2018; Radchuk et al. 2012). Cytokinins are important regulators involved in nearly all aspects of plant growth and development processes. The main panicle of the *Osckx2* mutant was longer and had improved PBP, SBP, and significantly improved GNP (Table 1). The improved GNP in the *Osckx2* mutants was due to increased secondary branching of the panicles and an increased number of grains in the secondary branching. A similar result was observed in the *OsCKX2* repression lines in rice (Ashikari et al. 2005; Joshi et al. 2018). In contrast to WT, the *Osckx2* mutants showed improved total flag leaf area due to the increase in both leaf length and leaf width (Table 1). The finding confirms that the loss of function of *OsCKX2* led to improved cytokinin content in leaf tissue, which in turn improved leaf area in both directions through active cell division. The uppermost flag leaf is metabolically active and decisive for grain yield (Sakamoto et al. 2006). The flag leaf increases light uptake for photosynthesis and nitrogen storage for grain filling and it redirects maximum photosynthetic energy to shrink tissue. Grain yield-related traits were positively associated with flag leaf area (Ashrafuzzaman, et al. 2009). The increased grain yield in the *Osckx2* mutant can be related to the improved reproductive (spikelet) structure in *Osckx2,* as more shrink tissue and maximum photosynthetic carbon are diverted from the flag leaves for grain filling.

The *Osckx2* improves grain morphology. Compared to WT, the *Osckx2* mutant appeared to be longer in grain length and slightly broader (Table 1). The variation in grain architecture has not been reported in previous reports (Ashikari et al. 2005; Joshi et al. 2018). Yeh et al (2015) found that *OsCKX2* downregulated rice lines resulted in a significant increase in grain weight. Similarly, it has reported that upregulation of cytokinin transport genes improves grain size in rice (Xiao et al. 2019). Our results confirmed previously reported results regarding improved grain length due to the loss of function of the *Osckx2* mutation in rice (Tsago et al. 2020). Studies on the cellular organization of rice grain revealed that the loss of function of *OsCKX2* led to an increase in the number and size of parenchyma cells in the spikelet hull of the *Osckx2* mutant (Tsago et al. 2020). A similar observation was also reported by different groups in rice (Li et al. 2011; Song et al. 2007; Sun et al. 2016). Further, it was reported that, compared to WT, the transcript levels of cell division regulating genes such as *OsCDKB2*, *OsCYCD2*, and *OsCYCA1* were up-regulated in the panicle tissue of the *Osckx2* mutant (Tsago et al. 2020). Thus,

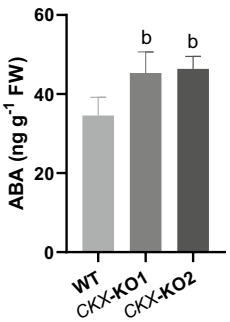

Fig. 8 Effects of the *Osckx2* mutation on ABA levels in leaf tissue. *Osckx2* mutant showed enhanced ABA level in leaf tissue compared to WT plant. Data ($n=3$) mean ± SD. Significant differences between the groups (WT and *CKX2*-KO) increase at $p \leq 0.01$ (b)

the study underscores that improved endogenous cytokinin content could promote cell division, proliferation, and differentiation (Jameson and Song 2016; Kyozuka 2007; Panda et al. 2018; Sakakibara 2006). Therefore, the improved grain length in the *Osckx2* mutant is due to the upregulation of genes involved in cell proliferation, which could be attributed to the increased cytokinin content in the *Osckx2* mutant. Several studies have shown that rice with poor grain filling is linked to lower cytokinin levels (Panda et al. 2018). The application of exogenous cytokinin to rice seeds resulted improved seed setting rate in rice plant (Pan et al. 2013). In the early stages of grain development, cytokinin enhances the division of cells, which facilitates the filling of grains (Mizutani et al. 2010). In addition, this cytokinin promotes nutrient transport into the developing endosperm (Rijavec et al. 2009; Hwang et al. 2012). The loss of *OsCKX2* resulted 16–23% higher grain yield in the MTU1010 rice background. In addition, we observe that improved grain yield is associated with the increased number of secondary branches in the panicle. According to studies in diverse rice backgrounds, repression or knockout of *OsCKX2* improved grain yield at different rates (Chen et al. 2020). Similarly, gene editing has been used to target mutations in *Gn1a* of the rice cultivar Zhonghua 11, a popularly grown modern japonica rice cultivar (Li et al. 2016).

Osckx2 mutants improved drought tolerance and maintained a cooler canopy through reduced transpiration rate

Generally, plants adapt to drought by reducing transpiration water loss as a first tire defense against dehydration stress (Fang and Xiong 2015). Our study demonstrated that, compared with WT leaves, excised *Osckx2* mutant leaves showed significantly lower water losses and maintained greater relative water content (Fig. 4). Further, both *Osckx2-1* (10.4%) and *Osckx2-2* (11.3%) transpire significantly less water than their WT counterparts when grown in hydroponic conditions (Fig. 4). Early-stage seedlings can serve as an effective assay system to establish drought tolerance. Under mimicked drought stress, withholding irrigation for 9 days, the *Osckx2* mutants maintained leaf turgidity and displayed improved tolerance. Moreover, *Osckx2* mutant rice seedlings recovered rapidly following reirrigation, showing an improved survival rate of 66–74% (Fig. 3). Conversely, WT rice seedlings were completely wilted and dried out under the same conditions and showed a 15–21% survival rate during the recovery phase (Fig. 3). Our findings indicate that the *Osckx2* mutant transpires water significantly more slowly and conserves the plant's water status. Alternatively, WT controls transpire more water and deplete the available water rapidly, making them vulnerable to drought. Because of minimal transpiration, *Osckx2* mutants are expected to preserve plant water status, which can be helpful for plant development at later stages under drought conditions. The improved survival rates (66–74%) of *Osckx2* mutants under prolonged drought stress may be attributed to this mechanism (Fig. 3).

Transpiration cooling protects plants from physiologically unacceptable canopy temperatures; otherwise, it inhibits or delays critical physiological processes (Deva et al. 2020). Canopy temperature depression (CTD) is an indicator of total plant water status that is a variation from the actual ambient temperature (Bazzer and Purcell 2020). The canopy temperature depression (CTD) is positively correlated with plant stress tolerance. In response to varying soil moisture conditions, the plant canopy temperature varied from 6 °C cooler to 7 °C warmer than the ambient temperature (Siebert et al. 2014). Our study indicates that the *Osckx2* mutant has a comparable lower thermal canopy capacity to WT rice under water-limiting conditions (5th day drought condition). Due to lower transpiration water loss, *Osckx2* mutants are able to maintain improved leaf water potential and delay the onset of dehydration stress, as indicated in the thermal imaging (Fig. 3l-m).

Osckx2 mutant maintained improved photosynthesis under physiological drought conditions

Photosynthetic mechanisms are highly conserved among green plants, and therefore, genetic manipulation is the most effective strategy for improving crop photosynthetic efficiency. The novel *Osckx2* allele may provide insights into future climate-resilient crops, by improving crop photosynthesis under physiological drought stress conditions. On the 5th day of physiological drought condition, the Ci, gs, and E parameters were significantly decreased in *Osckx2* mutants compared to WT (Fig. 5). The results indicate that in *Osckx2* plants, decreased water loss mechanisms including transpiration and conductance, resulted in conserved internal water, and plants showed physiological tolerance. Moreover, the *A*, pn/ci, and WUE were significantly improved in *Osckx2* mutants compared to the WT plant (Fig. 5). The improved photosynthesis efficiency of the *Osckx2* mutant directly indicates a better adaptation mechanism under dehydration conditions. Food security requires rice cultivars that use water more efficiently, therefore, optimizing soil water use for growth and optimum yield are fundamental traits to improve water use efficiency (Bertolino et al. 2019).

Osckx2 mutant confers cellular protection through improved antioxidant system

Drought stress often causes reactive oxygen species (ROS) to be generated and cause damage to cells, biomolecules, and

photosynthesis machinery (Achary et al. 2012). Our study indicated that *Osckx2* mutants exhibit improved antioxidant levels (CAT, APX, and SOD) under both control and water-deficit conditions (Fig. 6). ROS generation is neutralized by both enzymatic and non-enzymatic systems, maintains redox equilibrium within cells, and protects plant cells from oxidative damage (Achary et al. 2012). *Osckx2* exhibits improved levels of cellular antioxidants, which can be correlative with reduced levels of ROS accumulation under drought conditions (Fig. 6). Further, the *Osckx2* mutant plants showed improved membrane stability, as demonstrated by reduced electrolytic leakage and lipid peroxidation compared to WT (Fig. 6). Reduced leaf water potential and increased leaf relative water content (RWC) were considered suitable biomarkers to determine tissue dehydration, which positively correlated with drought tolerance (Zhang et al. 2019). Compared to its corresponding WT control rice, the *Osckx2* mutant rice plants showed improved leaf RWC (Fig. 4 c, d). Previous findings have also shown that drought-tolerant plants lose less water and have higher water use efficiency under water-stressed conditions than their non-tolerant counterparts (Santhosh Kumar et al. 2020). Improved leaf RWC assists in the accumulation of solutes that are compatible with intracellular cells, such as proline, glycine betaine, free amino acids, and soluble sugars, thereby protecting plant functional constituents, including proteins, enzymes, and genome integrity, thus enhancing cell function. In the present study, *Osckx2* mutants maintained higher RWC for longer periods of time during drought stress, which suggests a higher level of osmoregulation. In general, plants can conserve cellular hydration through osmotic adjustment under drought stress by causing a decrease in turgor that accumulates various osmolytes (Wang et al. 2019). Plants also activate their osmoprotection mechanism by increasing amino acids, sugars, proline, and glycine betaine to maintain their membrane integrity, as well as other macromolecules from ROS damage (Wang et al. 2019). The accumulation of proline is one of the most essential osmolytes to maintain cellular equilibrium during dehydration and salinity conditions. Osmolytes enhance membrane stability and cellular homeostasis; they neutralize and protect against the adverse effects of drought stress (Ghosh et al. 2022). In the *Osckx2* mutants, proline accumulation is significantly higher, which results in better membrane stability, resulting in the plants being able to maintain membrane integrity and cellular homeostasis and fully regain their physiological activity during the recovery period (Fig. 6) in contrast to the WT control plants. The *Osckx2* mutant rice plants maintained increased proline flux, which serves both as a molecular chaperone and a ROS scavenger. A similar adaptive mechanism has been reported previously in other plant systems. (Hong et al. 2000; Hayat et al. 2012; Furlan et al. 2020). This protection mechanism operated cumulatively and resulted in increased photosynthetic pigment content in *Osckx2* mutants, as shown in Fig. 6. In the present study, the improved photosynthetic pigment content under dehydration stress was also directly related to the improved photosynthetic efficiency of the *Osckx2* mutant (Fig. 5). In order to develop the correlation of current conservation, a CRISPR/Cas9-edited *Ospyl9* mutant rice showed a decrease in stomatal conductance, transpiration rate, and MDA content, but an increase in cuticle wax, panicle number, ABA content, antioxidants (CAT and SOD), and a higher survival rate (Usman et al. 2020).

OsCKX2-deficient mutants increased ABA accumulation in leaf

In plants, the phytohormone abscisic acid (ABA) serves as a drought sensor and is also essential for growth and development. ABA modulates various biochemical and physiological cascades such as seed germination, growth, osmotic adjustment, closure of stomata, root remodeling, senescence, and flowering during dehydration stress (Raghavendra et al. 2010; Yu et al. 2016). The indigenous level of ABA was improved in the *OsCKX2*-deficient mutant under normal condition (Fig. 8). Drought stress has led plants to evolve a variety of adaptation mechanisms, including protection and avoidance for survival. Gibberellic acid (GA) positively influences seed germination and plant development during seed germination and generative stages. Drought stress has a significant influence on GA levels in plants. Drought reduced GA content in maize has been reported (Wang et al. 2008). Osmotic and salt stress up-regulated GA biosynthesis genes *GA2ox6* and *GA2ox7* expression in *Arabidopsis* (Magome et al. 2008; Dubois et al. 2013). The present study speculates that the loss of function of the *OsCKX2*-deficient mutant might affect the level of indigenous GA, resulting in improved germination and root-shoot growth in *Osckx2* mutants. An in-depth investigation needs to be conducted in order to figure out how the mechanism works.

Conclusion

Cytokinin signaling has evolved as an intercellular communication network that is essential to crosstalk with other types of phytohormones and their regulating pathways in mediating plant stress responses. In the present study, we exploited gene editing to create a high-yielding mutant allele of *OsCKX2* in the indica rice cultivar. The loss of the *OsCKX2* allele improved water use efficiency by reducing transpiration and enhancing the antioxidant system. In this study, we gained a better understanding of how cytokinin plays a role in drought response pathways. Therefore, it is very important to understand the role of other members of the cytokinin oxidase family in the stress response

pathway. Genome editing has emerged as a tool to validate the role as well as the improvement of traits in many crops. Therefore, modifying specific members of cytokinin oxidase with the potential alteration of cytokinin in the crop is necessary in order to develop a high grain yield and climate-tolerant genotype to address future food security.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00299-024-03289-6.

Acknowledgements VMMA thanks the Council of Scientific and Industrial Research (CSIR), New Delhi, for awarding the Senior Research Associateship (SRA; Scientists Pool). We greatly appreciated the support provided by the metabolite facility at NIPGR for measuring cytokinin, auxin, and abscisic acid in plant samples.

Author contributions VMMA, MKR and MZA conceptualized the work. AR conducted the major experiments. VMMA analyzed the data and drafted the manuscript. SK, AR and HP were involved in the molecular confirmation edited lines. VMMA designed the experiments. VMMA, MKR, MZA, GP and PBM finalized the manuscript.

Funding This work was supported by research grant (NASF/CRISPR-Cas9-7003/2017–18) ICAR-National Agricultural Science Fund (NASF) awarded to MKR and VMMA.

Data availability The study includes original contributions that are detailed in the article or Supplementary Material. For further information, inquiries may be directed to the corresponding author.

Declarations

Conflict of interest The authors declare no conflict of interest.

References

Achary VMM, Patnaik AR, Panda BB (2012) Oxidative biomarkers in leaf tissue of barley seedlings in response to aluminum stress. Ecotoxicol Environ Saf 75:16–26. https://doi.org/10.1016/j.ecoenv.2011.08.015

Achary VMM, Reddy MK (2021) CRISPR-Cas9 mediated mutation in GRAIN WIDTH and WEIGHT2 (GW2) locus improves aleurone layer and grain nutritional quality in rice. Sci Rep 11:21941. https://doi.org/10.1038/s41598-021-00828-z

Akhtar SS, Mekureyaw MF, Pandey C et al (2020) Role of Cytokinins for Interactions of Plants With Microbial Pathogens and Pest Insects. Front Plant Sci 10:1777. https://doi.org/10.3389/fpls.2019.01777

Ashikari M, Sakakibara H, Lin S, et al (2005) Cytokinin oxidase regulates rice grain production. Science. 309, 741–745. https://www.science.org/doi/https://doi.org/10.1126/science.1113373

Ashrafuzzaman M, Islam MR, Ismail MR et al (2009) Evaluation of six aromatic rice varieties for yield and yield contributing characters. Int J Agric Biol 11:616–620

Bailey-Serres J, Parker JE, Ainsworth EA et al (2019) Genetic strategies for improving crop yields. Nature 575:109–118. https://doi.org/10.1038/s41586-019-1679-0

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39:205–207. https://doi.org/10.1007/bf00018060

Bazzer SK, Purcell LC (2020) Identification of quantitative trait loci associated with canopy temperature in soybean. Sci Rep 10:17604. https://doi.org/10.1038/s41598-020-74614-8

Bedada LT, Seth MS, Runo SM et al (2016) Drought tolerant tropical maize (*Zea mays L.*) developed through genetic transformation with isopentenyltransferase gene. Afr J Biotechnol 15:2447–2464. https://doi.org/10.5897/ajb2016.15228

Bertolino LT, Caine RS, Gray JE (2019) Impact of Stomatal Density and Morphology on Water-Use Efficiency in a Changing World. Front Plant Sci 10:225. https://doi.org/10.3389/fpls.2019.00225

Chen L, Zhao J, Song J, Jameson PE (2020) Cytokinin dehydrogenase: a genetic target for yield improvement in wheat. Plant Biotechnol J 18:614–630. https://doi.org/10.1111/pbi.13305

Deva CR, Urban MO, Challinor AJ et al (2020) Enhanced Leaf Cooling Is a Pathway to Heat Tolerance in Common Bean. Front Plant Sci 11:19. https://doi.org/10.3389/fpls.2020.00019

Dubois M, Skirycz A, Claeys H et al (2013) Ethylene Response Factor6 acts as a central regulator of leaf growth under water-limiting conditions in Arabidopsis. Plant Physiol 162:319–332. https://doi.org/10.1104/pp.113.216341

Fang Y, Xiong L (2015) General mechanisms of drought response and their application in drought resistance improvement in plants. Cell Mol Life Sci 72:673–689. https://doi.org/10.1007/s00018-014-1767-0

Furlan AL, Bianucci E, Giordano W et al (2020) Proline metabolic dynamics and implications in drought tolerance of peanut plants. Plant Physiol Biochem 151:566–578. https://doi.org/10.1016/j.plaphy.2020.04.010

Ghosh A, Shah MNA, Jui ZS et al (2018a) Evolutionary variation and expression profiling of Isopentenyl transferase gene family in *Arabidopsis thaliana L.* and *Oryza sativa L.* Plant Gene 15:15–27. https://doi.org/10.1016/j.plgene.2018.06.002

Ghosh S, Watson A, Gonzalez-Navarro OE et al (2018b) Speed breeding in growth chambers and glasshouses for crop breeding and model plant research. Nat Protoc 13:2944–2963. https://doi.org/10.1038/s41596-018-0072-z

Ghosh UK, Islam MN, Siddiqui MN et al (2022) Proline, a multifaceted signalling molecule in plant responses to abiotic stress: understanding the physiological mechanisms. Plant Biol 24:227–239. https://doi.org/10.1111/plb.13363

Gu B, Zhou T, Luo J et al (2015) An-2 Encodes a Cytokinin Synthesis Enzyme that Regulates Awn Length and Grain Production in Rice. Mol Plant 8:1635–1650. https://doi.org/10.1016/j.molp.2015.08.001

Hai NN, Chuong NN, Tu NHC et al (2020) Role and Regulation of Cytokinins in Plant Response to Drought Stress. Plants 9:422. https://doi.org/10.3390/plants9040422

Hayat S, Hayat Q, Alyemeni MN et al (2012) Role of proline under changing environments: a review. Plant Signal Behav 7:1456–1466. https://doi.org/10.4161/psb.21949

Hiscox JD, Israelstam GF (1979) A method for the extraction of chlorophyll from leaf tissue without maceration. Can J Bot 57:1332–1334. https://doi.org/10.1139/b79-163

Hluska T, Dobrev PI, Tarkowská D et al (2016) Cytokinin metabolism in maize: Novel evidence of cytokinin abundance, interconversions and formation of a new trans-zeatin metabolic product with a weak anticytokinin activity. Plant Sci 247:127–137. https://doi.org/10.1016/j.plantsci.2016.03.014

Hong Z, Lakkineni K, Zhang Z (2000) Removal of feedback inhibition of D1 pyrroline 5 carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiol 122:1129–1136. https://doi.org/10.1104/pp.122.4.1129

Huang M, Shan S, Cao J et al (2020) Primary-tiller panicle number is critical to achieving high grain yields in machine-transplanted

hybrid rice. Sci Rep 10:2811. https://doi.org/10.1038/s41598-020-59751-4
Hwang I, Sheen J, Muller B (2012) Cytokinin signaling networks. Annu Rev Plant Biol 63:353–380. https://doi.org/10.1146/annurev-arplant-042811-105503
Jameson PE, Song J (2016) Cytokinin: a key driver of seed yield. J Exp Bot 67:593–606. https://doi.org/10.1093/jxb/erv461
Joshi R, Sahoo KK, Tripathi AK et al (2018) Knockdown of an inflorescence meristem-specific cytokinin oxidase – OsCKX2 in rice reduces yield penalty under salinity stress condition. Plant Cell Environ 41:936–946. https://doi.org/10.1111/pce.12947
Joshi S, Choukimath A, Isenegger D et al (2019) Improved Wheat Growth and Yield by Delayed Leaf Senescence Using Developmentally Regulated Expression of a Cytokinin Biosynthesis Gene. Front Plant Sci 10:1285. https://doi.org/10.3389/fpls.2019.01285
Kant S, Burch D, Badenhorst P (2015) Regulated Expression of a Cytokinin Biosynthesis Gene IPT Delays Leaf Senescence and Improves Yield under Rainfed and Irrigated Conditions in Canola (*Brassica napus L.*). PLOS ONE 10:e0116349. https://doi.org/10.1371/journal.pone.0116349
Kumar MN, Verslues PE (2014) Stress physiology functions of the Arabidopsis histidine kinase cytokinin receptors. Physiol Plant 154:369–380. https://doi.org/10.1111/ppl.12290
Kuppu S, Mishra N, Hu R et al (2013) Water-Deficit Inducible Expression of a Cytokinin Biosynthetic Gene IPT Improves Drought Tolerance in Cotton. PLoS ONE 8:e64190. https://doi.org/10.1371/journal.pone.0064190
Kurakawa T, Ueda N, Maekawa M (2007) Direct control of shoot meristem activity by a cytokinin-activating enzyme. Nature 445:652–655. https://doi.org/10.1038/nature05504
Kuroha T, Tokunaga H, Kojima M (2009) Functional Analyses of LONELY GUY Cytokinin-Activating Enzymes Reveal the Importance of the Direct Activation Pathway in Arabidopsis. Plant Cell 21:3152–3169. https://doi.org/10.1105/tpc.109.068676
Kyozuka J (2007) Control of shoot and root meristem function by cytokinin. Curr Opin Plant Biol 10:442–446. https://doi.org/10.1016/j.pbi.2007.08.010
Le DT, Nishiyama R, Watanabe Y (2011) Genome-Wide Expression Profiling of Soybean Two-Component System Genes in Soybean Root and Shoot Tissues under Dehydration Stress. DNA Res 18:17–29. https://doi.org/10.1093/dnares/dsq032
Le DT, Nishiyama R, Watanabe Y et al (2012) Identification and Expression Analysis of Cytokinin Metabolic Genes in Soybean under Normal and Drought Conditions in Relation to Cytokinin Levels. PLoS ONE 7:e42411. https://doi.org/10.1371/journal.pone.0042411
Li YB, Fan CC, Xing YZ (2011) Natural variation in GS5 plays an important role in regulating grain size and yield in rice. Nat Genet 43:1266–1269. https://doi.org/10.1038/ng.977
Li M, Li X, Zhou Z et al (2016) Reassessment of the Four Yield-related Genes Gn1a, DEP1, GS3, and IPA1 in Rice Using a CRISPR/Cas9 System. Front Plant Sci 7:377. https://doi.org/10.3389/fpls.2016.00377
Li S, Zhao B, Yuan D (2013) Rice zinc finger protein DST enhances grain production through controlling *Gn1a/OsCKX2* expression. Proc Natl Acad Sci USA 110:3167–3172. https://doi.org/10.1073/pnas.1300359110
Liu Z, Yanxia Lv, Zhang M et al (2013) Identification, expression, and comparative genomic analysis of the IPT and CKX gene families in Chinese cabbage (*Brassica rapa ssp. pekinensis*). BMC Genomics 14:594. https://doi.org/10.1186/1471-2164-14-594
Lu Y, Chuan M, Wang H et al (2022) Genetic and molecular factors in determining grain number per panicle of rice. Front Plant Sci 13:964246. https://doi.org/10.3389/fpls.2022.964246
Lubovská Z, Dobrá J, Storchová H et al (2014) Cytokinin oxidase/dehydrogenase overexpression modifies antioxidant defense against heat, drought and their combination in Nicotiana tabacum plants. J Plant Physiol 171:1625–1633. https://doi.org/10.1016/j.jplph.2014.06.021
Magome H, Yamaguchi S, Hanada A (2008) The DDF1 transcriptional activator upregulates expression of a gibberellin- deactivating gene, GA2ox7, under high-salinity stress in Arabidopsis. Plant J 56:613–626. https://doi.org/10.1111/j.1365-313X.2008.03627.x
Mai W, Abliz B, Xue X (2021) Increased Number of Spikelets per Panicle Is the Main Factor in Higher Yield of Transplanted vs. Direct-Seeded Rice Agronomy 11:2479. https://doi.org/10.3390/agronomy11122479
Merewitz EB, Du H, Yu W et al (2011) Elevated cytokinin content in ipt transgenic creeping bentgrass promotes drought tolerance through regulating metabolite accumulation. J Exp Bot 63:1315–1328. https://doi.org/10.1093/jxb/err372
Mizutani M, Naganuma T, Tsutsumi K et al (2010) The syncytium-specific expression of the Orysa; KRP3 CDK inhibitor: implication of its involvement in the cell cycle control in the rice (*Oryza sativa L.*) syncytial endosperm. J Exp Bot 61:791–798. https://doi.org/10.1093/jxb/erp343
Nguyen KH, Van Ha C, Nishiyama R (2016) Arabidopsis type B cytokinin response regulators ARR1, ARR10, and ARR12 negatively regulate plant responses to drought. Proc Natl Acad Sci USA 113:3090–3095. https://doi.org/10.1073/pnas.1600399113
Nishiyama R, Watanabe Y, Fujita Y (2011) Analysis of Cytokinin Mutants and Regulation of Cytokinin Metabolic Genes Reveals Important Regulatory Roles of Cytokinins in Drought, Salt and Abscisic Acid Responses, and Abscisic Acid Biosynthesis. Plant Cell 23:2169–2183. https://doi.org/10.1105/tpc.111.087395
Nishiyama R, Watanabe Y, Leyva-Gonzalez MA (2013) Arabidopsis AHP2, AHP3, and AHP5 histidine phosphotransfer proteins function as redundant negative regulators of drought stress response. Proc Natl Acad Sci USA 110:4840–4845. https://doi.org/10.1073/pnas.1302265110
Ogonowska H, Barchacka K, Gasparis S et al (2019) Specificity of expression of TaCKX family genes in developing plants of wheat and their co-operation within and among organs. PLoS ONE 14:e0214239–e0214239. https://doi.org/10.1371/journal.pone.0214239
Pan SG, Rasul F, Li W et al (2013) Roles of plant growth regulators on yield, grain qualities and antioxidant enzyme activities in super hybrid rice (Oryza sativa L). Rice 6:10. https://doi.org/10.1186/1939-8433-6-9
Panda BB, Sekhar S, Dash SK et al (2018) Biochemical and molecular characterisation of exogenous cytokinin application on grain filling in rice. BMC Plant Biol 18:89. https://doi.org/10.1186/s12870-018-1279-4
Peleg Z, Reguera M, Tumimbang E et al (2011) Cytokinin-mediated source/sink modifications improve drought tolerance and increase grain yield in rice under water-stress. Plant Biotechnol J 9:747–758. https://doi.org/10.1111/j.1467-7652.2010.00584.x
Pospíšilová H, Jiskrová E, Vojta P et al (2016) Transgenic barley overexpressing a cytokinin dehydrogenase gene shows greater tolerance to drought stress. New Biotechnol 33:692–705. https://doi.org/10.1016/j.nbt.2015.12.005
Qin H, Gu Q, Zhang J et al (2011) Regulated Expression of an Isopentenyltransferase Gene (IPT) in Peanut Significantly Improves Drought Tolerance and Increases Yield Under Field Conditions. Plant Cell Physiol 52:1904–1914. https://doi.org/10.1093/pcp/pcr125
Radchuk V, Radchuk R, Pirko Y et al (2012) A somaclonal line SE7 of finger millet (*Eleusine coracana*) exhibits modified cytokinin homeostasis and increased grain yield. J Exp Bot 63:5497–5506. https://doi.org/10.1093/jxb/ers200

Raghavendra AS, Gonugunta VK, Christmann A et al (2010) ABA perception and signalling. Trends Plant Sci 15:395–401. https://doi.org/10.1016/j.tplants.2010.04.006

Reape TJ, McCabe PF (2008) Apoptotic-like programmed cell death in plants. New Phytol 180:13–26. https://doi.org/10.1111/j.1469-8137.2008.02549.x

Reguera M, Peleg Z, Abdel-Tawab YM et al (2013) Stress-Induced Cytokinin Synthesis Increases Drought Tolerance through the Coordinated Regulation of Carbon and Nitrogen Assimilation in Rice. Plant Physiol 163:1609–1622. https://doi.org/10.1104/pp.113.227702

Rijavec T, Kovac M, Kladnik A et al (2009) A comparative study on the role of cytokinins in caryopsis development in the maize miniature 1 seed mutant and its wild type. J Integr Plant Biol 51:840–849. https://doi.org/10.1111/j.1744-7909.2009.00863.x

Sakakibara H (2006) Cytokinins: activity, biosynthesis, and translocation. Annu Rev Plant Biol 57:431–449. https://doi.org/10.1146/annurev.arplant.57.032905.105231

Sakamoto T, Morinaka Y, Ohnishi T et al (2006) Erect leaves caused by brassinosteroid deficiency increase biomass production and grain yield in rice. Nat Biotechnol 24:105–109. https://doi.org/10.1038/nbt1173

Santosh Kumar VV, Verma RK, Yadav SK et al (2020) CRISPR-Cas9 mediated genome editing of drought and salt tolerance (OsDST) gene in indica mega rice cultivar MTU1010. Physiol Mol Biol Plants 26:1099–1110. https://doi.org/10.1007/s12298-020-00819-w

Siebert S, Ewert F, Rezaei EE et al (2014) Impact of heat stress on crop yield—on the importance of considering canopy temperature. Environ Res Lett 9:044012. https://iopscience.iop.org/article/https://doi.org/10.1088/1748-9326/9/4/044012

Song XJ, Huang W, Shi M et al (2007) A QTL for rice grain width and weight encodes a previously unknown RING-type E3 ubiquitin ligase. Nat Genet 39:623–630. https://doi.org/10.1038/ng2014

Sun PY, Zhang WH, Wang YH (2016) OsGRF4 controls grain shape, panicle length and seed shattering in rice. J Integr Plant Biol 58:836–847. https://doi.org/10.1111/jipb.12473

Susan J, Fatemeh R, Latifeh P (2013) Effect of abiotic stresses on histidine kinases gene expression in Zea mays L. cv. SC. 704. J Stress Physiol Biochem 9:124–135. http://www.jspb.ru/issues/2013/N1/JSPB_2013_1_124-135.pdf

Tsago Y, Chen Z, Cao H et al (2020) Rice gene, OsCKX2-2, regulates inflorescence and grain size by increasing endogenous cytokinin content. Plant Growth Regul 92:283–294. https://doi.org/10.1007/s10725-020-00637-w

Tu B, Tao Z, Wang S et al (2022) Loss of Gn1a/OsCKX2 confers heavy-panicle rice with excellent lodging resistance. J Integr Plant Biol 64:23–38. https://doi.org/10.1111/jipb.13185

Usman B, Nawaz G, Zhao N et al (2020) Precise Editing of the OsPYL9 Gene by RNA Guided Cas9 Nuclease Confers Enhanced Drought Tolerance and Grain Yield in Rice (Oryza sativa L) by Regulating Circadian Rhythm and Abiotic Stress Responsive Proteins. Int J Mol Sci 21:7854. https://doi.org/10.3390/ijms21217854

Vadassery J, Reichelt M, Hause B et al (2012) CML42-mediated calcium signaling coordinates responses to Spodoptera herbivory and abiotic stresses in Arabidopsis. Plant Physiol 159:1159–1175. https://doi.org/10.1104/pp.112.198150

Verslues PE, Agarwal M, Katiyar-Agarwal S et al (2006) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. Plant J 45:523–539. https://doi.org/10.1111/j.1365-313x.2005.02593.x

Vojta P, Kokáš F, Husičková A et al (2016) Whole transcriptome analysis of transgenic barley with altered cytokinin homeostasis and increased tolerance to drought stress. New Biotechnol 33:676–691. https://doi.org/10.1016/j.nbt.2016.01.010

Wang C, Yang A, Yin H et al (2008) Influence of water stress on endogenous hormone contents and cell damage of maize seedlings. J Integr Plant Biol 50:427–434. https://doi.org/10.1111/j.1774-7909.2008.00638.x

Wang X, Mao Z, Zhang J et al (2019) Osmolyte accumulation plays important roles in the drought priming induced tolerance to post-anthesis drought stress in winter wheat (Triticum aestivum L). Environ Exp Bot 166:103804. https://doi.org/10.1016/j.envexpbot.2019.103804

Wang Y, Liu H, Xin Q (2014) Genome-wide analysis and identification of cytokinin oxidase/dehydrogenase (CKX) gene family in foxtail millet (*Setaria italica*). Crop J 2:244–254. https://doi.org/10.1016/j.cj.2014.05.001

Werner T, Nehnevajova E, Köllmer I et al (2010) Root-Specific Reduction of Cytokinin Causes Enhanced Root Growth, Drought Tolerance, and Leaf Mineral Enrichment in Arabidopsis and Tobacco. Plant Cell 22:3905–3920. https://doi.org/10.1105/tpc.109.072694

Xiao XO, Zeng YM, Cao BH et al (2017) PSAG12-IPT overexpression in eggplant delays leaf senescence and induces abiotic stress tolerance. J Hortic Sci Biotechnol 92:349–357. https://doi.org/10.1080/14620316.2017.1287529

Xiao Y, Liu D, Zhang G et al (2019) Big Grain3, encoding a purine permease, regulates grain size via modulating cytokinin transport in rice. J Integr Plant Biol 61:581–597. https://doi.org/10.1111/jipb.12727

Xing Y, Zhang Q (2010) Genetic and molecular bases of rice yield. Annu Rev Plant Biol 61:421–442. https://doi.org/10.1146/annurev-arplant-042809-112209

Xu Y, Burgess P, Zhang X et al (2016) Enhancing cytokinin synthesis by overexpressing ipt alleviated drought inhibition of root growth through activating ROS-scavenging systems in Agrostis stolonifera. J Exp Bot 67:1979–1992. https://doi.org/10.1093/jxb/erw019

Xu Y, Huang B (2017) Transcriptional factors for stress signaling, oxidative protection, and protein modification in ipt-transgenic creeping bentgrass exposed to drought stress. Environ Exp Bot 144:49–60. https://doi.org/10.1016/j.envexpbot.2017.10.004

Yeh S-Y, Chen H-W, Ng C-Y et al (2015) Down-Regulation of Cytokinin Oxidase 2 Expression Increases Tiller Number and Improves Rice Yield. Rice 8:36. https://doi.org/10.1186/s12284-015-0070-5

Yin C, Zhu Y, Li X et al (2021) Molecular and genetic aspects of grain number determination in rice (Oryza sativa L). Int J Mol Sci 22:728. https://doi.org/10.3390/ijms22020728

Yu LH, Wu SJ, Peng YS et al (2016) Arabidopsis EDT1/HDG11 improves drought and salt tolerance in cotton and poplar and increases cotton yield in the field. Plant Biotechnol J 14:72–84. https://doi.org/10.1111/pbi.12358

Zalabák D, Pospíšilová H, Šmehilová M et al (2013) Genetic engineering of cytokinin metabolism: prospective way to improve agricultural traits of crop plants. Biotechnol Adv 31:97–117. https://doi.org/10.1016/j.biotechadv.2011.12.003

Zalewski W, Galuszka P, Gasparis S et al (2010) Silencing of the HvCKX1 gene decreases the cytokinin oxidase/dehydrogenase level in barley and leads to higher plant productivity. J Exp Bot 61:1839–1851. https://doi.org/10.1093/jxb/erq052

Zhang F, Zhou G (2019) Estimation of vegetation water content using hyperspectral vegetation indices: a comparison of crop water indicators in response to water stress treatments for summer maize. BMC Ecol 19:18. https://doi.org/10.1186/s12898-019-0233-0