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ORIGINAL ARTICLE

Rice *CENTRORADIALIS 2* regulates seed germination and salt tolerance via ABA-mediated pathway

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Abstract

Key message **A FT/TFL1 subfamily gene, rice *CENTRORADIALIS* 2, also known as RCN1, regulates seed germination and increase salt tolerance via ABA-mediated pathway. The ABA synthesis and metabolism related genes were changed relative expression levels.**

Abstract Seed germination is a complex biological process that is affected by many factors. Although a number of germination-related genes have been reported, the molecular mechanism of germination regulation has not yet been fully elucidated. Here, we reported that the rice *OsCEN2* gene can negatively regulate seed germination. The germination speed of *OsCEN2*-RNAi seeds was significantly faster while that of *OsCEN2*-overexpression (OE) seeds was slower than that of the wild type (WT). The results of qRT-PCR showed that the *OsCEN2* expression was increased in the early stage of seed germination. Exogenous application of abscisic acid (ABA) on seeds and seedlings showed that *OsCEN2*-OE seeds and seedlings were highly sensitive to ABA during germination and post-germination growth, respectively. The determination of endogenous ABA content in seeds also showed that the ABA content of *OsCEN2*-RNAi seeds was lower, while that of *OsCEN2*-OE seeds was higher. Moreover, the transgenic plants changed salt tolerance because of the altered ABA level. In addition, differences were also observed in the expression of genes related to ABA synthesis and metabolism in the seeds of *OsCEN2*-transgenic lines. This study reveals that *OsCEN2* regulates the germination speed by affecting the content of ABA during seed germination and provides a theoretical basis for research on rice direct seeding.

Introduction

During seed germination, vigorous seeds absorb water under suitable environmental conditions, expand their volume, release dormancy, until the embryo breaks through the seed coat, which is key to the life cycle of all flowering plants that propagate through seeds (Nonogaki et al. 2010). The difference in germination speed of crop seeds affects the uniformity of emergence and the regularity of seedlings, which in turn affects crop yields (Gubler et al. 2005). In rice, seed germination speed and regularity also affect mechanical direct seeding, leading to problems with sparse seedling of direct seeding rice, as well as low or unstable yields (Mahender et al. 2015).

In the process of seed germination, environmental factors play a vital role. In addition, endogenous factors such as seed developmental stage, hormone levels and seed structure are also of importance. Studies have indicated that gibberellin (GA) and abscisic acid (ABA) are mutually antagonistic during seed dormancy and germination; ABA can inhibit while GA can promote seed germination (Corbineau et al. 2014). Seeds can maintain the dormancy state through accumulating ABA during development, avoiding preharvest sprouting. During seed germination, the ABA content would gradually decrease, and exogenous application of ABA would

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inhibit seed germination to a certain extent (Gubler et al. 2005; Jiang et al. 2019; Song et al. 2020).

ABA signal transduction is mainly composed of three core components, including the ABA receptor (REGULATORY COMPONENT OF ABA RECEPTOR 1/PYRABACTIN RESISTANCE 1/PYR1-RELATED HOMOLOGUES, RCAR/PYR1/PYL), protein phosphatase (PROTEIN PHOSPHATASE 2C, PP2C) and SNF1-related kinase 2 (SNF1-RELATED PROTEIN KINASE 2, SnRK2) (Miyazono et al. 2009). Under normal growth conditions, ABA is sensed by receptors such as RCAR/PYR1/PYL, which interacts with the free-state PP2C (with protein phosphatase activity) and forms a complex, PYR/RCAR-PP2C, to inhibit PP2C activity. Afterward, SnRK2 is phosphorylated to activate downstream transcription factors responding to ABA signals (Finkelstein 2013; Koramutla et al. 2021). ABA INSENSITIVE 5 (ABI5) is a major transcription factor in the ABA signaling pathway. In rice, the *abi5* mutant was not sensitive to ABA during germination, but plants overexpressing *ABI5* were highly sensitive to ABA (Zou et al. 2008). ABI5 can also bind to the ABA response element (ABRE) on the promoter of its target genes to promote or inhibit their expression. In *Arabidopsis*, ABI5 can bind to the promoters of *EARLY METHIONINE-LABELED 1* (*EM1*) and *EM6* to regulate seed germination (Carles et al. 2002). *ABI3/OsVP1* is mainly involved in signal transduction during plant seed development and thus affects seed germination. Rice OsVP1 can interact with Rc and another regulatory factor, OsC1, to enhance the sensitivity of seeds to ABA by promoting the biosynthesis of proanthocyanidins and the perception of ABA signals, ultimately inhibiting early germination (Wang et al. 2020).

OsCEN2 belongs to the *FLOWERING LOCUS T* (*FT*)*/TERMINAL FLOWER 1* (*TFL1*) subfamily within the phosphatidylethanolamine binding protein (PEBP) family (Coelho et al. 2014). In addition to playing a role in regulating flowering time and plant structure, *FT/TFL1* is also associated with plant seed germination (Karlgrenet et al. 2011; Jin et al. 2021). A study has revealed that the *Arabidopsis FT* gene can inhibit the expression of the *FLOWERING LOCUS C* (*FLC*) gene by activating the RNA-COOLAIR of *FLC* and then release seed dormancy by regulating the chromatin state (Chen et al. 2018). However, another study reported that *FT* inhibits the *FLC* expression through a 5′ promoter region of *FLC* (Luo et al. 2019). The potato (*Solanum tuberosum*) *FT/TFL1* family gene *StCEN* plays a regulatory role in the process of potato tuberization. The expression level of *StCEN* is related to the growth rate of buds, which is lower in *StCEN* overexpression lines compared to the wild type (Morris et al. 2019). In poplars (*Populus* spp.), reducing the expression of *PopCEN1* shortens the dormancy time and accelerates the flowering time, while the trees overexpressing *PopCEN1* show the opposite phenotype and the flowering of which is completely inhibited (Mohamed et al. 2010). The *PcFT2* gene of pear can promote plant vegetative growth, and delay seed dormancy and leaf senescence (Freiman et al. 2015). In *Arabidopsis*, *AtMFT* regulates seed germination through ABA and GA signaling pathways (Xi et al. 2010). Many studies have shown that the loss-of-function *mft* mutant exhibits sensitivity to ABA during seed germination. In the process of seed germination, the expression of *MFT* is directly regulated by two key transcription factors, ABI3 and ABI5, in the ABA signaling pathway. At the same time, MFT directly inhibits *ABI5*, negatively regulating ABA signaling to promote embryo growth (Xi et al. 2010). In wheat (*Triticum aestivum*), the increased *TaMFT* expression is related to low germination index. Transient expression of *TaMFT* in immature embryos can inhibit the premature germination of immature embryos, indicating that TaMFT is an inhibitor of seed germination (Nakamura et al. 2011). In rice, *OsMFT2*-knockout lines show preharvest sprouting, while *OsMFT2*-overexpression lines exhibit delayed sprouting. It is revealed that OsbZIP23, OsbZIP66 and OsbZIP72 can interact with OsMFT2; in addition, OsbZIP23/66/72 can bind to the promoter of the ABA response element gene *Rab16A*. Such binding is enhanced by *OsMFT2*, indicating that *OsMFT2* negatively regulates seed germination (Song et al. 2020). The functions of multiple *FT/TFL1* family genes have been reported; however, whether *OsCEN2* can affect rice seed germination remains elucidated.

In this study, a rice *CENTRORADIALIS* gene *OsCEN2*, also known as *RCN1*, is responsible for regulating seed germination in rice. We found that *OsCEN2*-RNAi seeds showed increased germination speed, while *OsCEN2*-overexpression (OE) seeds showed delayed germination. Exogenous application of ABA to the seeds and seedlings revealed that *OsCEN2* affected seed germination and the sensitivity of seedlings to ABA. In summary, *OsCEN2* affects the ABA content during seed germination by affecting the expression of genes related to ABA synthesis and metabolism, which in turn affects the seed germination speed.

Materials and methods

Plant material and growth conditions

Zhonghua 11 (ZH11, *O. sativa* ssp. Japonica) used as WT and recipient for genetic transformation in this study. The plants were grown in the paddy field at South China Agricultural University in Guangzhou. Rice cultivation is managed according to local common practices.

To construct the *OsCEN2* overexpression vector, 522-bp fragment of the OsCEN2 cDNA sequence driven by the Ubiquitin (Ubi) promoter was inserted into the pYLox vector (Yu et al. 2010).

To construct the *OsCEN2* RNAi vector, two fragments of *OsCEN2* were isolated by PCR using the primers RNAi1 and RNAi2 from the rice ZH11 genome DNA into the *Eco*R I–*Hin*d III sites and from immature panicle cDNA with primers RNAi2 and RNAi3 into the *Hin*d III–*Sal* I sites of the cloning vector of pBluescript II. The RNAi construct was driven by the native promoter of *OsCEN2*. The *OsCEN2* promoter was amplified from the rice genome using the primers PRNAiF and PRNAiR and cloned into the *Bam*H I–*Eco*R I sites of the cloning vector pBluescript II with RNAi fragments. After sequencing, the total fragments containing the native promoter and RNAi fragments were digested with *Bam*H I and *Sal* I and cloned into the binary vector pCAMBIA1380. The cloning was performed following the previously described methods (Li et al. 2011; Zhou et al. 2014).

To generate the CRISPR/Cas9 vector, the CEN2Cas were constructed using pYLgRNA-OsU3, as described previously (Ma et al. 2015). The sequence of the target site was 5′- TGT GTCTAAACCAAGAGTTG-3′, which contained a protospacer adjacent motif (PAM) AGG at the 3′ end.

All constructs were confirmed by sequencing, introduced into Agrobacterium tumefaciens EHA105 cells and transformed into ZH11 by Agrobacterium-mediated transformation according our previously reported method (Jiang et al. 2018). All primers used for vector construction are listed in Table S1.

RNA isolation and qRT-PCR

Total RNA was isolated from the seed using fruit-mate (TaKaRa, Dalian, China) and RNAiso Plus (TaKaRa, Dalian, China). The seeds were ground into powder (100 mg) in liquid nitrogen, then transferred to 500 µL fruit-mate and mixed thoroughly. After centrifugation, the supernatant fluid was transferred and mixed with 500 µL RNAiso Plus reagent and mixed thoroughly and incubated for 5 min. Then 200 µL chloroform was added and mixed thoroughly. After centrifugation, the aqueous phase was transferred and mixed with an equal volume of isopropanol. After gently inversions, the mixture was chilled at −20 °C for 20 min and then centrifuged. The supernatant fluid should be removed and washed using 75% ethanol with diethyl pyrocarbonate-treated ddH_2O (DEPC-ddH_2O). The precipitate is dissolved with DEPC-ddH_2O and quantified with a DU730 spectrophotometer (Beckman Coulter, Germany).

cDNA was synthesized with a 5 × iScript RT Supermix kit (TaKaRa, Dalian, China) using about 2 µg of total RNA.

qRT-PCR was performed with SYBR premix Ex Taq II (TaKaRa, Dalian, China) in a total volume of 20 µL on the Bio-Rad CFX 96 following the manufacturer's protocol. Data were normalized to the internal rice UBIQUITIN (UBI) gene, and relative quantification was used for data analysis. All primers for qRT-PCR are shown in Supporting Information Table S1.

Measurement of ABA level

Seeds freshly harvested were dehulled and soaked in distilled water at 28 °C. Seeds (100 mg) at 0, 6 and 12 HAI (hours after imbibition) were ground into fine powder in liquid nitrogen. They were used for endogenous ABA measurement by plant ABA ELISA Kit (Kejing Biological Technology Co. Ltd.) following the manufacturer's protocol.

Seed germination assay and ABA or FLU treatment

To treat with ABA or Fluridone (FLU), the fresh harvest grains were soaked with distilled water, 5 µM ABA or 100 µmol/L FLU solution for 24 h. A total of 180 seeds were spread onto plates covered with wet filter papers for three biological replicates of each line. The plates were placed in a chamber at 28 °C.

For counting the germination rates of grains, 180 seeds for each line were soaked in distilled water for 24 h and then spread onto plates covered with wet filter papers for three biological replicates.

To medium culture with water and agar in plate, 60 husked seeds for each line were sterilized with 1.5% NaClO for 20 min and washed with sterilized water five times. The seeds were spread onto the medium for three biological replicates of each line.

Germination was defined as the emergence of the radical, and the number of germinated seeds was counted every 3 or 6 h. The germination rate was calculated as the number of total germinated seeds divided by the number of total seeds. The photographs were taken by camera and the length of roots and shoots were measured by calipers and ruler.

To treat with ABA, the 10 d-seedlings were cultured with hydroponics with or without 5 µM ABA solution. To determine the ABA sensitivity of post-gemination growth, 45 husked seeds of each line were sterilized with 1.5% NaClO and washed with sterilized water for three times. The seeds were spread onto 1/2 MS medium. We conducted the germination assay every 12 h to select the seeds with the same growth vigor and state. Then, they were transferred onto 1/2 MS medium containing a gradient concentration of ABA (0, 1, 3, 5, 10 µM). For every treatment, three biological replicates were conducted. The seeds were grown in the chamber at 28 ℃. The length of roots and shoots was measured at 10 days after transplanting.

RNA-seq and data analysis

Total RNA was extracted from seeds in triplicate at 0, 6 and 12 HAI (hour after imbibition) using fruit-mate and RNAiso

Plus (TaKaRa, Dalian, China), following to the protocol. RNA was used for RNA-seq by BGI Genomics (Wuhan, China), using the DNBSEQ platform.

Salt treatments

WT and transgenic homozygous seeds were germinated in water and transferred to Kimura B nutrient solution after germination 4 days. For salt stress conditions, 2-week-old seedlings were treated with Kimura B complete nutrient solution supplemented with 150 mM sodium chloride. After stress treatments for 6 days, seedlings were transplanted to Kimura B nutrient solution for 6 days.

Relative water content

The relative water content measurement was performed according to the reported methods (Khan et al. 2017). Three mature leaves at 0 and 3rd day of salt stress were harvested, and the fresh weight (FW) was recorded. These leaf samples were kept 2 h in water to attain turgidity and measured turgid weight (TW). These leaves were kept in oven at 60 °C for 24 h and measured the dry weight (DW). The relative water content was calculated as the following formula, RWC (%) = [(FW − DW) / (TW − DW)] × 100%

The electrolyte leakage

The electrolyte leakage measurement was performed following the reported methods (Lv et al. 2017). In brief, three leaves from three plants at 0 and 6th day of salt stress were cut into segments of the same size and immersed in 20 mL of double distilled water in a 250-mL test tube for 6 h with shaking. The initial conductivity (R1) was measured with a conductivity meter (Model DDS-11A, Shanghai Hongyi Instruments and Apparatuses Co. Ltd.). Then, the test tubes with leaf segments were placed in boiling water for 30 min and cooled naturally to room temperature, and the conductivity (R2) was determined. The relative electrolyte leakage was calculated as the ratio of R1 to R2.

Data analysis

To test statistically significant differences in the results, the one-way ANOVA (LSD analysis) test was conducted pairwise comparisons between transgenic line and wild type. These tests were performed using SPSS version 20. We judged the data significance level according to the *P* value. In all statistical tests, *P* value < 0.05 was considered statistically significant. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

Accession numbers

Sequence data from this article can be found in the Rice Annotation Project database (RAP-DB), under the following accession numbers: OsCEN2 (Os11g0152500), OsNCED3 (Os03g0645900), OsNCED5 (Os12g0617400), OsZEP1 (Os04g0448900), ABA8ox1 (Os02g0703600).

Results

OsCEN2 affects seed germination speed

To study the function of *OsCEN2* during seed germination, we obtained *OsCEN2* overexpression (*OsCEN2*-OE) plants and RNAi (*OsCEN2*-RNAi) plants of rice cultivar 'Zhonghua 11'. Seed germination experiments showed that the germination speed of *OsCEN2* transgenic seeds was significantly different from that of wild type (WT). The *OsCEN2*-RNAi plants (RNAi12 and RNAi2) germinated significantly faster while the *OsCEN2*-OE plants (OE1 and OE2) germinated slower compared to WT at 48 h after imbibition (HAI; Fig. 1a). To further explore the effect of *OsCEN2* on seed germination, we calculated the germination speed of these seeds. At 24 HAI, the WT and *OsCEN2*-RNAi seeds began to germinate, while the *OsCEN2*-OE seeds did not. At 36 HAI, the OE1 seeds began to germinate, while the OE2 seeds begin to germinate until 45 HAI. The germination rate of WT seeds had reached 18.9%, while the germination rates of the two *OsCEN2*-RNAi lines were 29.8% and 28.9%, respectively, at 45 HAI. The germination rates of RNAi lines were 57.05%, WT was 38.01%, while those of OE1 and OE2 were 23.71% and 15.08% at 57 HAI. The germination rate of *OsCEN2*-RNAi and WT seeds exceeded 90% at 99 HAI, while those of OE1 and OE2 were 78.74 and 83.34%, respectively (Fig. 1b). In addition, we conducted germination experiments under sterile conditions. The germination speed of *OsCEN2*-RNAi seeds was faster than that of WT, while that of *OsCEN2*-OE lines was the slowest (Fig. S1), which is consistent with the trend of germination speed of seeds with husks (Fig. 1b). The germination experiments conducted on *OsCEN2*-knockout seeds also showed that the germination speed of transgenic seeds was significantly faster than that of WT seeds (Fig. S2a, c). This is consistent with the results obtained from *OsCEN2*-RNAi seeds.

At 23 d post-germination, a significant difference was observed in plant height between WT and transgenic seedlings (Fig. 1c). The plant height of the two *OsCEN2*-RNAi lines was 9.53 and 9.96 cm, respectively, which were significantly higher than that of 8.34 cm of WT. On the contrary, the plant height of the two *OsCEN2*-OE lines was

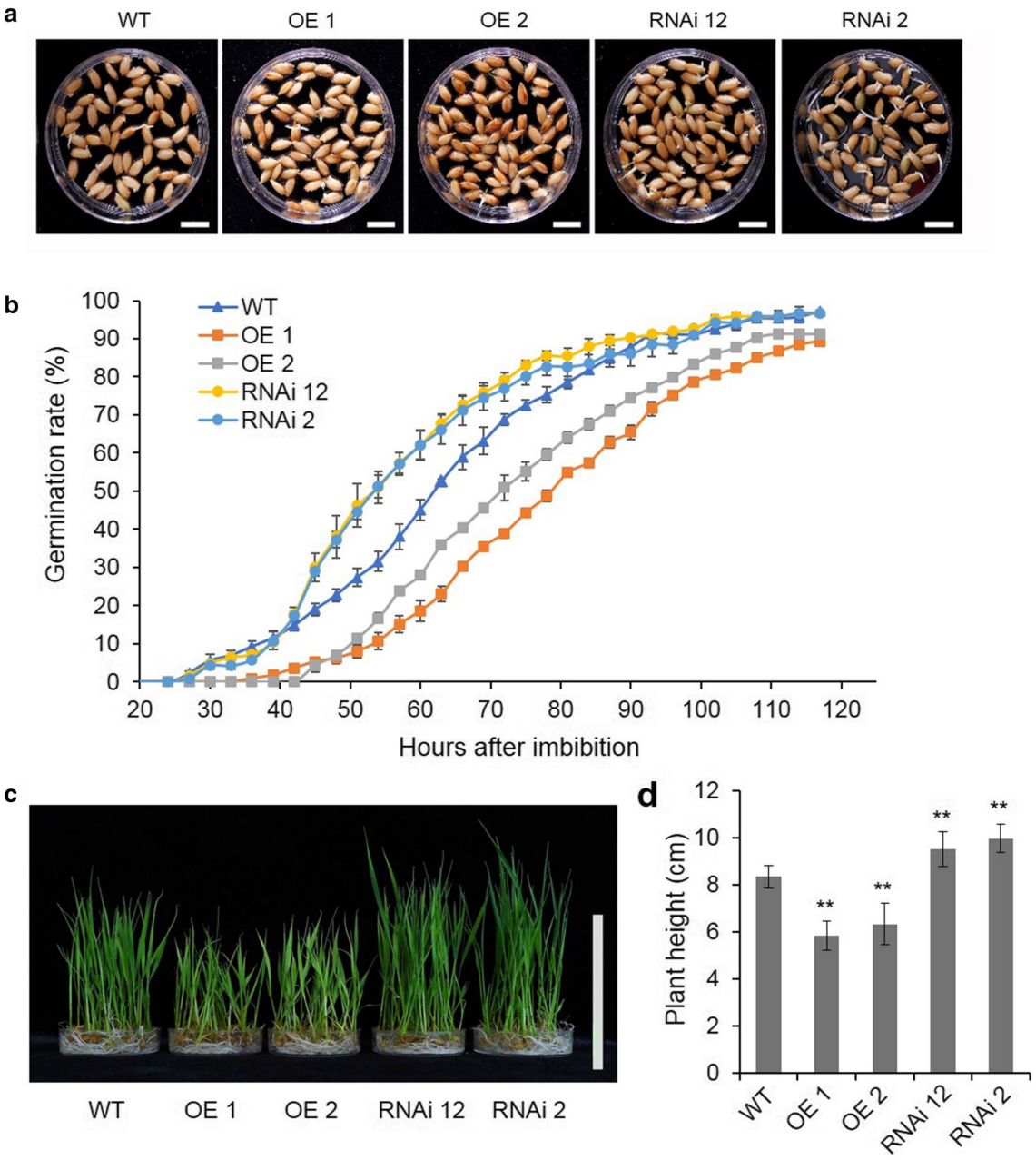

Fig. 1 OsCEN2 negatively regulates seed germination. **a** Phenotype of WT, overexpression and RNAi lines at 48 h after imbibition. Scale bars: 1 cm. **b** Grains germination rate of WT, overexpression and RNAi lines. The grains were soaked in distilled water and the germination grains were counted every 3 h after 24 h imbibition. Values are presented as means ± SD of three biological replicates ($n = 60$). **c** Seedling phenotypes of WT, overexpression and RNAi lines. Scale bar: 10 cm. **d** The plant height of WT, overexpression and RNAi lines. Values are presented as means ± SD ($n = 30$), $^{**}P < 0.01$; one-way ANOVA (LSD analysis) test. WT: wild type Zhonghua 11; OE 1, OE 2: overexpression lines; RNAi 12, RNAi 2: RNAi lines

5.85 and 6.34 cm, respectively, which were significantly lower than that of WT (Fig. 1d). Moreover, the *OsCEN2*-knockout seedlings were also significantly higher than WT (Fig. S2b, d).

Expression pattern of *OsCEN2*

We used qRT-PCR to investigate the expression level of *OsCEN2* during seed germination. The results showed that *OsCEN2* was expressed throughout the germination process of rice seeds (Fig. 2a). With seed germination proceeded, the expression level of *OsCEN2* was gradually increased and

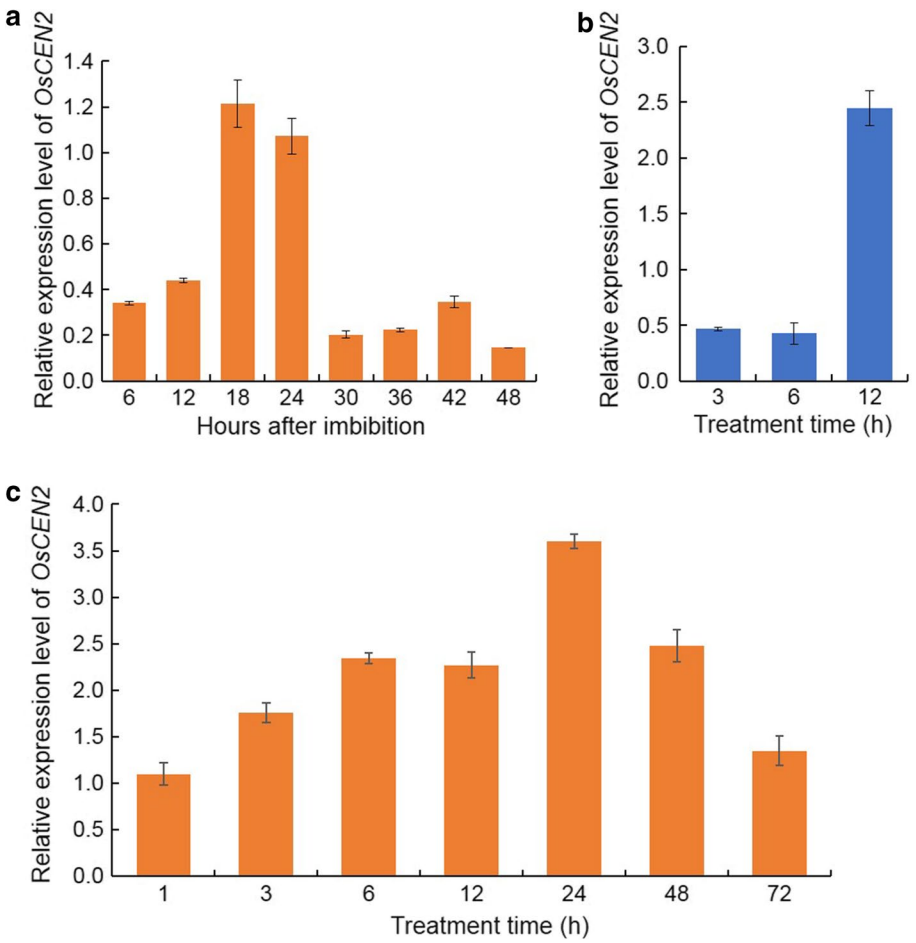

Fig. 2 Expression pattern analysis of *OsCEN2*. **a** Expression levels of *OsCEN2* at different time during seed germination. **b** Expression levels of *OsCEN2* at 3, 6 and 12 h time points of germinated seeds after 5 μM ABA solution treatment. **c** Expression levels of *OsCEN2* at different time of 10 d-seedling after 5 μM ABA treatment. Values are presented as means ± SD ($n = 3$)

reached the peak at 18 HAI. Then, the expression level of *OsCEN2* began to decline sharply at 24 HAI and remained a low level at 30–48 HAI (Fig. 2a).

ABA is a key hormone that regulates seed germination. To study the effect of ABA on *OsCEN2*, we examined the *OsCEN2* expression in germinated seeds after 5 μM ABA treatment. The results of qRT-PCR showed that the expression level of *OsCEN2* increased significantly after 12 h of ABA treatment (Fig. 2b). In addition, we applied ABA on 10-day-old WT rice seedlings, and the expression level of *OsCEN2* increased with the increase in treatment duration, reached the highest level at 24 h and then began to decline (Fig. 2c). The above results indicate that exogenous ABA application can induce the expression of *OsCEN2*.

OsCEN2 affects rice sensitivity to ABA

To study the effect of exogenous ABA application on seed germination, we treated the seeds with water and 5 μM ABA solution, respectively. The results showed that after ABA treatment, the seed germination speed exhibited delay (Fig. 3a, S3b). Under ABA treatment conditions, the RNAi12 seeds showed a relative lower sensitivity compared with WT seeds. While the OE2 seeds showed a higher sensitivity compared to WT seeds (Fig. 3a). For example, at time point 112 HAI, the germination rate of WT seeds decreased from 68.3% to 51.7% after ABA treatment, while the RNAi12 seeds decreased from 78.9% to 76.7%, the OE1 seeds decreased from 27.2% to 5.6% (Fig. 3a). The above results suggest that ABA has a greater impact on the germination speed of *OsCEN2* OE seeds compared to WT and RNAi seeds. As for the final germination rate, WT and *OsCEN2*-RNAi seeds changed little, while that of *OsCEN2*-OE seeds decreased by 21.1% after ABA treatment (Fig. 3a), suggesting that the *OsCEN2*-OE seeds are more sensitive to ABA.

We further observed the effect of exogenous ABA application on the post-germination growth of seedlings. In this study, seeds were treated with water and ABA solution with different concentrations, respectively. The results showed that the decrease in plant height of *OsCEN2*-OE seedlings was significantly greater than that of WT and *OsCEN2*-RNAi seedlings (Fig. 3b, c), and the root length also showed similar patterns (Fig. 3b, d). These results indicate that in the process of post-germination growth, *OsCEN2*-RNAi

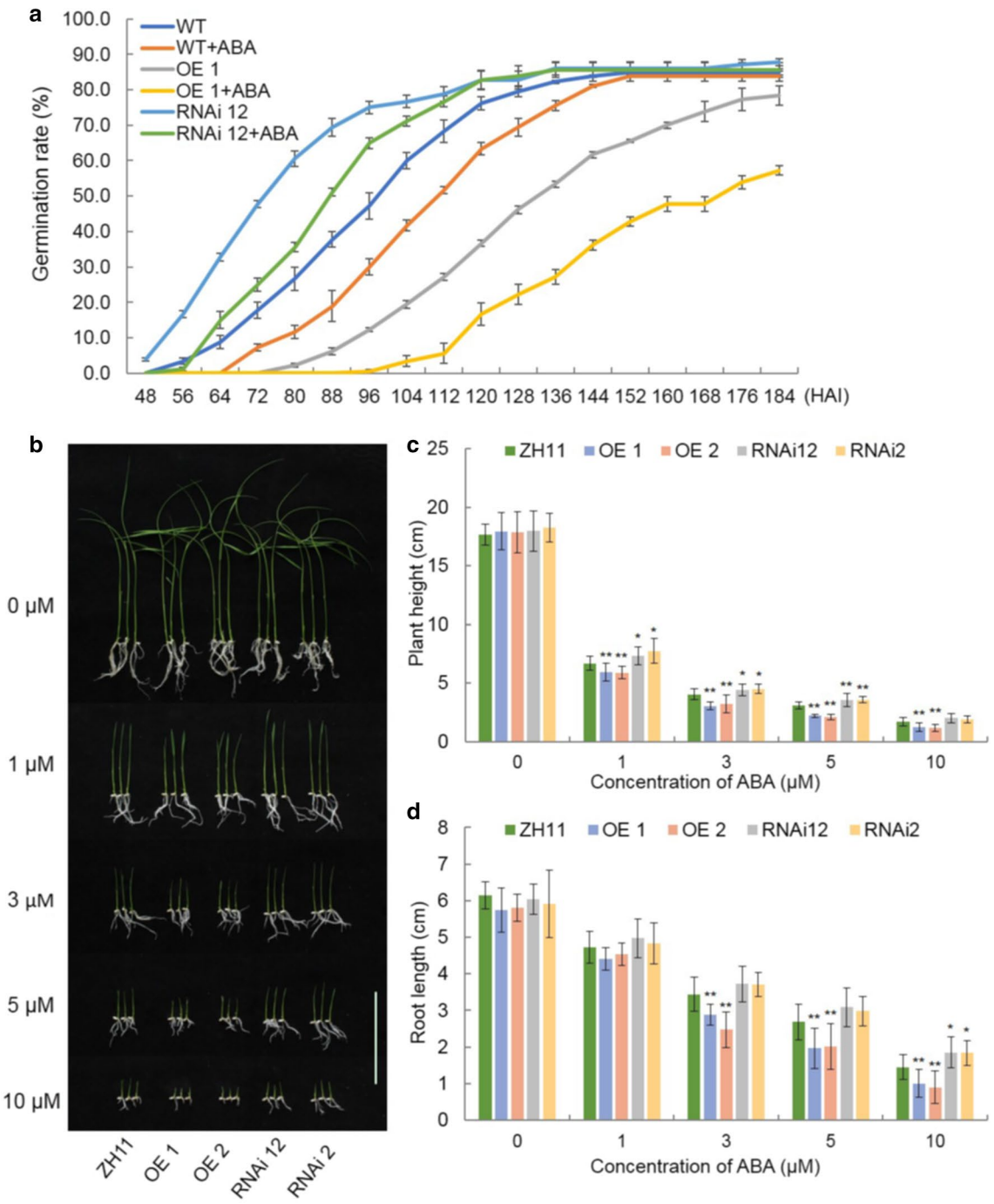

Fig. 3 ABA treatment influences seed germination and seedling growth. **a** Dynamic seed germination rate of WT, OE 1 and RNAi 12. The grains were soaked in distilled water, with or without 5 μM ABA, which were harvested at the same season. The germination rate was counted every 6 h after 48 h-imbibition. Values are presented as means ± SD of three biological replicates ($n = 60$). **b** Phenotypes of WT, OE 1, OE 2, RNAi 12 and RNAi 2 of germinated seeds under different ABA level treatments for 10 days. Scale bar: 10 cm. **c**, **d** The plant height (**c**) and root length (**d**) of WT, OE 1, OE 2, RNAi 12 and RNAi 2 of germinated seeds under different ABA level treatments for 10 days in **b**. Values are presented as means ± SD ($n = 30$). *: $P < 0.05$; **: $P < 0.01$; one-way ANOVA (LSD analysis) test. WT: wild type Zhonghua 11; OE 1, OE 2: overexpression lines; RNAi 12, RNAi 2: RNAi lines

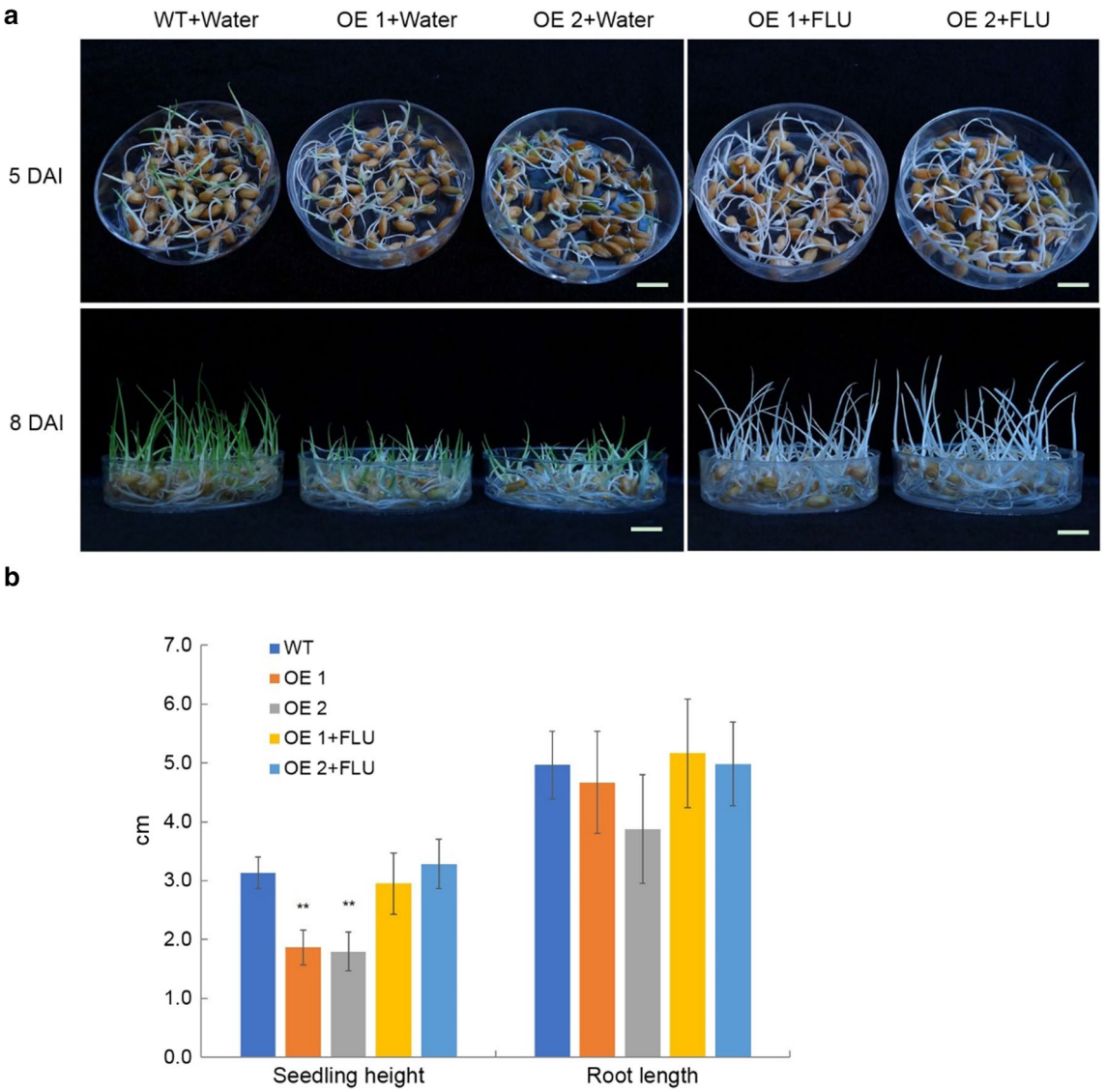

Fig. 4 Effects of FLU on rice seed germination and seedling growth. **a** Germinated seeds phenotypes with water or FLU treated for 5 and 8 days separately. Scale bars: 1 cm. **b** The seedling height and root length of treatment in **a** for 8 days. Values are presented as means ± SD ($n = 30$), $^{**}P < 0.01$; the one-way ANOVA (LSD analysis) test. WT: wild-type Zhonghua 11; OE 1, OE 2: overexpression lines. FLU: fluridone

seedlings are less sensitive, while *OsCEN2*-OE seedlings are more sensitive to ABA compared with WT.

Changes in endogenous ABA content of transgenic rice seeds lead to varied seed germination speeds

ABA in plants is mainly synthesized through the carotenoid pathway. Fluridone (FLU) can inhibit the biosynthesis of carotenoids, thereby affecting the ABA content in plants. After germination, the growth of *OsCEN2*-OE seedlings was significantly slower than WT seedlings. However, no significant difference was observed between *OsCEN2*-OE and WT seedlings after OE seeds treatment with 100 µmol/L FLU (Fig. 4a). At 8 DAI, there was no obvious difference between FLU-treated *OsCEN2*-OE and WT seedlings from phenotype (Fig. 4a). The plant height was consistent among them too (Fig. 4b). However, significant difference was found in the growth rate between the *OsCEN2*-OE seedlings without FLU treatment and WT (Fig. 4a, b), indicating that the inhibition of endogenous ABA synthesis can accelerate *OsCEN2*-OE seed germination.

To further explore the reasons for the difference in seed germination, we examined the ABA content in the seeds. Before water soaking, the ABA content in *OsCEN2*-RNAi seeds was significantly lower than that in WT, while the ABA content in *OsCEN2*-OE seeds was the highest. At 6

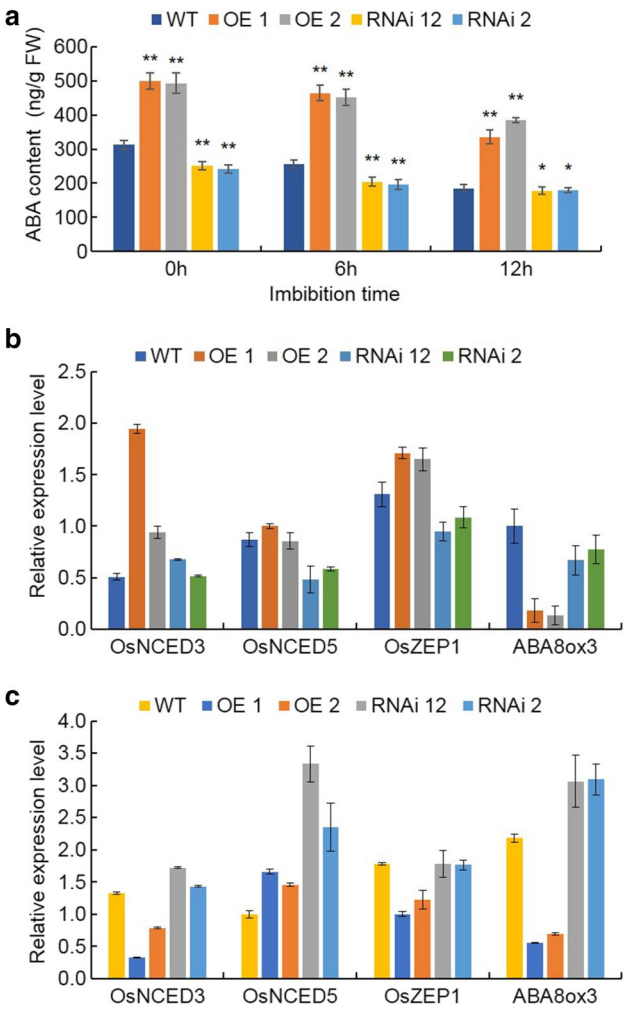

Fig. 5 ABA levels and relative expression levels of ABA relative genes during seed germination. **a** ABA content in the seeds after imbibition in water for 0, 6 and 12 h. Values are presented as means ± SD ($n = 3$), *: $P < 0.05$, **$P < 0.01$; the one-way ANOVA (LSD analysis) test. (b-c) Relative expression levels analysis of ABA relative genes in seeds before imbibition (**b**) and after imbibition for 12 h (**c**). WT: wild-type Zhonghua 11; OE 1, OE 2: overexpression lines; RNAi 12, RNAi 2: RNAi lines

HAI, the ABA content in all these lines were decreased compared to before imbibition, respectively. At this time, the ABA content in *OsCEN2*-OE seeds was still significantly higher than that of WT and *OsCEN2*-RNAi seeds (Fig. 5a). At 12 HAI, the ABA content of *OsCEN2*-OE seeds kept decreasing compared to at 6 HAI. However, the ABA content in *OsCEN2*-OE seeds was the highest among WT, overexpression and RNAi lines (Fig. 5a). The above results indicate that *OsCEN2* affects the ABA content in seeds, and we speculate that the change in ABA content is the main factor affecting seed germination.

Changes in the expression level of genes related to ABA synthesis and metabolism

To study the reasons for the changes in ABA content in seeds, we investigated the expression levels of ABA-related genes. The expression levels of the ABA synthesis-related genes *OsNCED3*, *OsNCED5* and *OsZEP1* were higher in *OsCEN2*-OE seeds, while lower in *OsCEN2*-RNAi seeds compared to WT before water soaking. The ABA degradation gene *ABA8ox3* was expressed at a low level in *OsCEN2*-OE seeds, but it was highly expressed in *OsCEN2*-RNAi seeds (Fig. 5b). At 12 HAI, the expression levels of *OsNCED3* and *OsZEP1* in *OsCEN2*-OE seeds were decreased and that of *ABA8ox3* was also lower compared to WT. The expression levels of *OsNCED5* and *ABA8ox3* in *OsCEN2*-RNAi seeds were higher than those of WT (Fig. 5c). These results indicate that *OsCEN2* can regulate the expression level of ABA-related genes, resulting in the change of ABA content.

Changes in the resistance of *OsCEN2*-transgenic plants to salt stress

The change of ABA content in plants affects their resistance to abiotic stress, such as drought and salt conditions. To confirm the effect of changes in ABA content on the resistance of *OsCEN2*-transgenic plants, the rice seedlings were treated with 150 mM NaCl solution. The results showed that the phenotype of *OsCEN2*-OE plants was no obvious different from WT before NaCl treatment, and no visible difference too in the phenotype after NaCl treatment (Fig. S4a-c). Although the relative electrolyte leakage (EL) also showed no significant difference between the *OsCEN2*-OE and WT plants before NaCl treatment, after salt treatment the EL value of WT was 22.91%, while that of the two *OsCEN2*-OE lines was 6.47% and 6.33%, respectively (Fig. S4d), indicating that *OsCEN2*-OE lines are more resistant to salt stress than WT. In addition, the *OsCEN2*-knockout plants all died at 7 d of recovery after NaCl treatment, while most of WT plants resumed growth (Fig. 6a–c). The *OsCEN2*-knockout plants had significantly higher relative EL than WT, and they also exhibited a significantly lower relative water content compared to WT (Fig. 6d, e). This indicated that the knockout plants are more sensitive to salt tolerance than WT. The above results showed that *OsCEN2* expression level leads to change tolerance of rice to salt treatment. These results suggest *OsCEN2* maybe regulate ABA content and then influence the seedling resistance to salt stress.

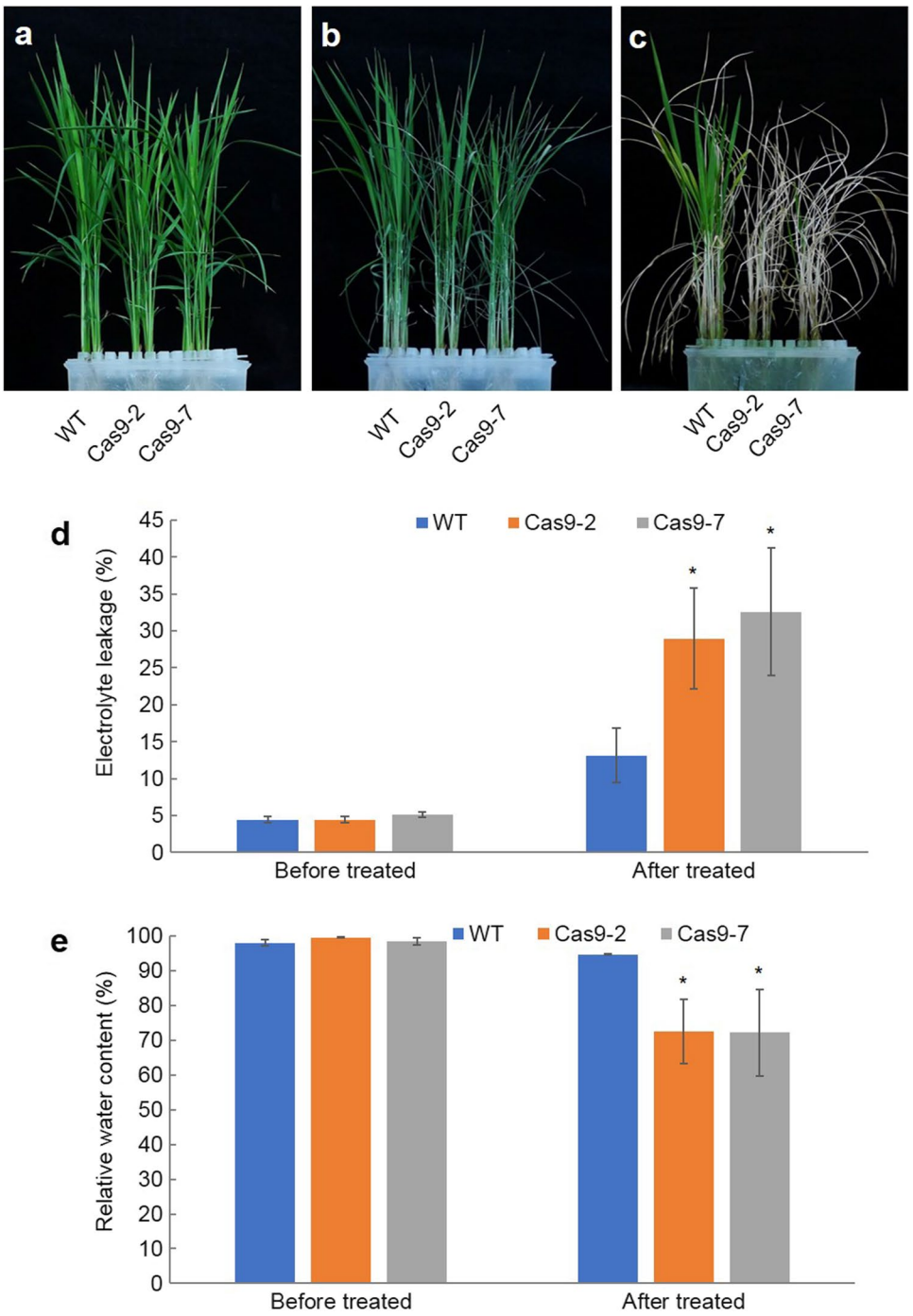

Fig. 6 *OsCEN2* knockout lines decreases salt stress tolerance. **a**–**c** Gross morphology of *OsCEN2* knockout lines and WT rice plants before treatment (**a**), after 150 mM NaCl treatment for 6 days (**b**) and recovered for 6 days **c** are shown. **d**–**e** Electrolyte leakage (**d**) and relative water content (**e**) of WT, *Cas9-2* and *Cas9-7* lines before and after salt treatment for 6 days and 3 days. Values are presented as means ± SD ($n = 9$), *: $P < 0.05$; one-way ANOVA (LSD analysis) test. WT: wild-type Zhonghua 11; *Cas9-2*, *Cas9-7*: *OsCEN2* knockout lines

OsCEN2 regulates plant hormone and MAPK signaling pathways during seed germination

Transcriptome (RNA-seq) helps to analyze the molecular regulation pathways of genes. We collected transcriptome data of rice seeds at 6, 12 and 24 HAI to investigate the changes in rice seeds responding to *OsCEN2* expression. Venn diagram analysis showed that a total of 1,104 differentially expressed genes (DEGs) were found at 12 HAI, while at 6 and 24 HAI, only 279 and 181 DEGs were identified (Fig. 7a); 58 and 97 DEGs were presented in OE and RNAi lines compared with WT at all the three time points, respectively (Fig. 7b). Gene ontology (GO) analysis showed that DEGs were enriched in starch and sugar metabolism, MAPK signaling pathway and glycolysis pathway at 6 HAI (Fig. 7c); DEGs were enriched in MAPK signaling pathway, plant hormone signal transduction, fatty acid metabolism and amino acid synthesis pathway at 12 HAI (Fig. 7d);

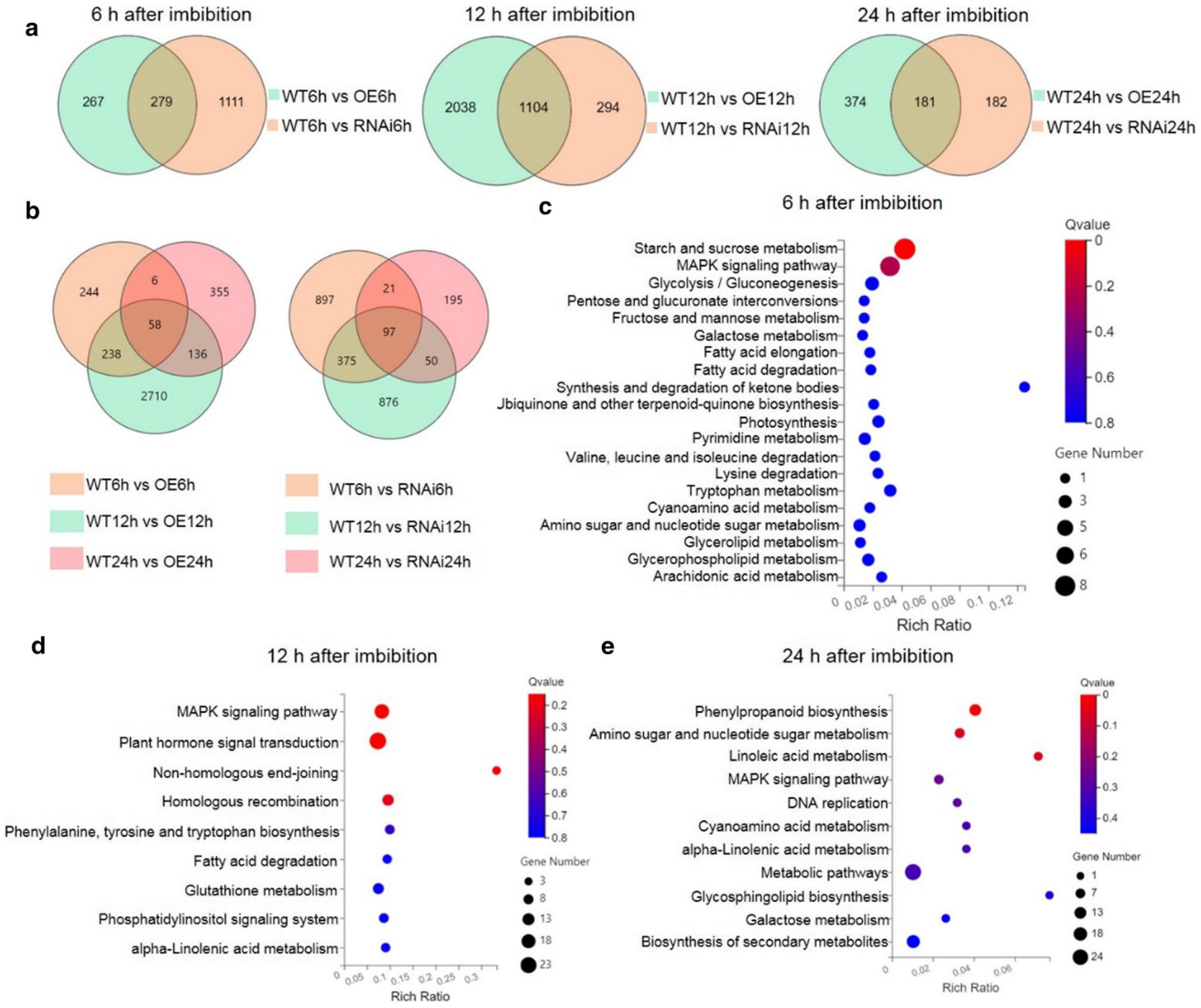

Fig. 7 DEGs at the 6, 12 and 24 h of seed imbibition are involved in plant hormone signal transduction and MAPK signal pathway. **a** Venn diagram showing DEGs overlap between OE lines (green plate) and RNAi lines (orange plate) relative to WT at the 6, 12 and 24 h after seed imbibition. **b** Venn diagram showing DEGs which expression levels were different from WT in *OsCEN2* overexpression lines or RNAi lines overlap at 6 h (orange plate), 12 h (green plate) and 24 h (pink plate) after seed imbibition. **c**–**e** Gene ontology enrichment pathways predicted to be involved in seed germination at the 6 h (**c**), 12 h (**d**) and 24 h (**e**) after seed imbibition. WT: wild-type Zhonghua 11; OE: overexpression plants; RNAi: RNAi plants

and DEGs were enriched in phenylpropanoid biosynthesis, MAPK signaling pathway and amino sugar and nucleotide sugar metabolism pathway at 24 HAI (Fig. 7e).

Among these DEGs, the expression trends of 73 DEGs were opposite between *OsCEN2*-OE and *OsCEN2*-RNAi lines compared with WT at 6 HAI, which were involved in plant hormone signaling, MAPK signaling pathway and sugar metabolism (Fig. S5a, S6). At 12 HAI, 81 DEGs that were involved in plant hormone signaling and starch and sugar metabolism showed opposite expression trends between *OsCEN2*-OE and *OsCEN2*-RNAi lines (Fig. S5b, S7). At 24 HAI, 92 DEGs that participated in plant hormone signaling and ABC transport exhibited different expression patterns between *OsCEN2*-OE and *OsCEN2*-RNAi lines (Fig. S5c, S8). The above results indicate that *OsCEN2* may regulate plant hormone signal transduction, sugar metabolism and MAPK signal pathways to regulate seed germination in the early stage of germination.

ABA and GA play different key roles in the regulation of seed dormancy and germination, and the metabolism and signaling by both phytohormones also changes during seed development (Shu et al. 2015). To illustrate the changes of ABA- and GA-related genes during germination, we analyzed the expression in transcriptome data and performed

the heat map analysis of ABA and GA-related genes at 6, 12 and 24 h after imbibition. The results showed the expression levels of several genes showed in opposite direction in *OsCEN2* overexpression and RNAi lines (Fig. S9). At 6 HAI, the expression level of *OsNF-YC5* (LOC112936059) and *ABA8ox1* (LOC9267503) decreased in overexpression plants, while increased in RNAi plants. At 12 HAI, *OsNF-YC5* (LOC112936059) and AAA-ATPase ASD (LOC4352916) decreased in overexpression plants, while increased in RNAi plants. In addition, *ethylene-responsive transcription factor ERF110* (LOC4351606), *PP2C37* (LOC9268838) and *zinc finger protein 2* (LOC4347837) were decreased in overexpression plants, while increased in RNAi plants (Fig. S9). These results suggested that *OsCEN2* affected the expression levels of ABA-related genes during germination.

We pay attention to the genes relative to GA, a heat map analysis was conducted at different timepoints. At 6 HAI, the expression level of *OsCPS2* (LOC9266189) was increased in overexpression plants, while decreased in RNAi plants. In contrary, *myb-related protein 308* (LOC4349938) was decreased in overexpression plants while increased in RNAi plants. *Senescence-specific cysteine protease SAG39* (LOC4335170) increased in overexpression plants while decreased in RNAi plants at 12 HAI. In addition, *gibberellin 2-beta-dioxygenase 6-like*, *GA2ox6* (LOC9266251) increased in overexpression plants while decreased in RNAi plants at 24 HAI. However, *cytochrome P450 714D1-like* (LOC4339131) and *OsGA20ox2* (LOC4325003) were decreased in overexpression plants while increased in RNAi plants. These genes show the opposite trend in overexpression plants and RNAi plants (Fig. S10). These results suggested that OsCEN2 affected the expression levels of GA-related genes during germination.

Discussion

OsCEN2 belongs to the *FT/TFL1* subfamily within the PEBP gene family, the members of which have been extensively studied (Coelho et al. 2014). Their functions involve dormancy (Chen et al. 2018), leaf senescence (Freiman et al. 2015) and flowering (Nakagawa et al. 2002). In this study, we found that *OsCEN2* could affect the germination of rice seeds. The germination of *OsCEN2*-OE seeds was delayed, while that of *OsCEN2*-RNAi seeds was accelerated, which is consistent with the results of other studies. For example, the *PcFT2* gene of pear can delay seed dormancy and leaf senescence (Freiman et al. 2015); *Arabidopsis AtMFT* regulates seed germination through ABA and GA signaling pathways (Xi et al. 2010); and *OsMFT2* negatively regulates seed germination by affecting the sensitivity of seeds to ABA during germination and post-germination growth in rice (Song et al. 2020).

There are many factors that affect the germination of rice seeds, including the inherent genetic characteristics of seeds such as α-amylase activity, endogenous plant hormones and soluble sugars, and external environmental factors such as temperature, humidity and light (An et al. 2018). In this study, the expression of *OsCEN2* was induced by ABA in both seeds and seedlings. *OsCEN2*-OE seeds were more sensitive to ABA during germination, while *OsCEN2*-RNAi seeds showed lower sensitivity to ABA, which indicates that *OsCEN2* plays an important role in ABA signal transduction. ABA and GA play different key roles in the regulation of seed dormancy and germination, and the metabolism and signaling by both phytohormones also changes during seed development (Shu et al. 2015). The effects of these hormones depend on both the amounts of one relative to the other, resulting from the rates of synthesis and catabolism, as well as the sensitivity of the tissues to these hormones (Finkelstein et al. 2008). Studies have shown that the sensitivity of plant seeds to ABA is the key to determine whether seeds can break dormancy and turn to the germination stage (Schmitz et al. 2002). In *Arabidopsis*, there are several ABA-insensitive (ABI) genes: *ABI1* and *ABI2* encode PP2C, *ABI3* encodes B3 transcription factor, *ABI4* encodes APETALA2-like transcription factor, and *ABI5* encodes bZIP transcription factor (Finkelstein and Lynch 2000; Merlot et al. 2001; Shu et al. 2013; Feng et al. 2014). These are all key genes in the plant ABA signaling pathway and play an important role in the biological processes of seed germination and dormancy and in response to adverse environments (Piskurewicz et al. 2008; Lopez-Molina et al. 2001, 2002). The *abi3*, *abi4* and *abi5* mutants are insensitive to ABA during seed germination and early seedling development and are lack of dormancy (Söderman et al. 2000; Zou et al. 2007; Feng et al. 2014). Studies have found that the *sapk2* mutant was ABA-insensitive in both germination and post-germination stages, indicating *SAPK2* plays a key role in ABA-mediated seed dormancy. Therefore, the seed germination process is highly associated with the sensitivity of seeds to ABA, which indicates that *OsCEN2* may regulate seed germination by affecting the sensitivity of seeds to ABA.

Studies have demonstrated that ABA content can affect seed germination speed (Jiang et al. 2019; Song et al. 2020). In the present study, the slow germination speed and low germination rate of *OsCEN2*-OE seeds maybe related to the ABA content in seeds. The ABA content in *OsCEN2*-OE dry seeds was higher than that in WT and *OsCEN2*-RNAi dry seeds. In addition, the germination rate and germination speed of *OsCEN2*-OE seeds were reduced more after treatment with exogenous ABA. In plants, ABA 8'-hydroxylation is the main pathway of ABA catabolism. In *Arabidopsis*, the CYP707A family members *CYP707A1-CYP707A4*

encode ABA 8'-hydroxylase; the seeds of *cyp707a2* mutant are highly dormant due to the high accumulation of ABA (Kushiro et al. 2004). ABI4 can positively regulate the synthesis of ABA in seeds to maintain the dormancy state of seeds (Shu et al. 2013). In this study, the expression levels of ABA synthesis-related genes in *OsCEN2*-OE dry seeds were higher while in *OsCEN2*-RNAi seeds were lower than those in WT; however, the expression pattern of the degradation gene *ABA8ox3* was the opposite. These results are consistent with those previously reported. Therefore, *OsCEN2* affects the endogenous ABA content by affecting the expression of ABA synthesis-related genes, thereby altering the germination rate and germination speed of seeds.

Published literatures reported that ABA modulates plant adaptation to osmotic stress mainly through increasing cellular dehydration tolerance and reducing water loss (Lee et al. 2012). The nine-cis-epoxycarotenoid dehydrogenases (NCEDs) are the key enzymes in ABA biosynthesis (Zhu et al. 2009). Some NCED proteins have been reported to contribute to increasing ABA levels and abiotic stress tolerance in plants (Sun et al. 2012). OsMADS23 was reported to confer drought and salt tolerance by regulating ABA biosynthesis in rice. More importantly, in parallel to *osmads23* mutant, *osnced2* mutants had reduced ABA accumulation and increased sensitivity to drought and oxidative stress (Li et al. 2021). Previous results showed that the *OsNAC2* overexpression plants with higher ABA contents exhibited increased drought and salt tolerance (Jiang et al. 2019). Consistent with these findings, our results also showed that the knockout lines with lower ABA levels are more sensitive to salt tolerance than WT, and the overexpression lines with higher ABA levels are more resistant to drought and salt stress than WT. These results suggest *OsCEN2* maybe regulate ABA content and then influence the seedling's resistance to drought and salt stress.

This study provides a basis for altering the germination rate and speed of rice seeds as well as their resistance to salt stress through regulation of ABA content. It is of great significance for variety breeding suit for mechanical direct seeding with appropriate germination speed and increased salt tolerance.

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Author Contribution Statement CZ and DJ designed the research. YH and WC performed most experiments and analyzed experimental data. JT, XL, YZ, XG and JY conducted a part of experiments. HY wrote the manuscript, CZ and DJ revised the paper. All authors approved the final manuscript.

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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