1 **Rice annexin OsANN4 interacting with OsCDPK24, reduces root**
2 **aerenchyma formation by modulating H₂O₂ under ABA**
3 **treatment**

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21
22 Date of submission: 2019/1/12

23 Number of figures: 7

24 Word count: 5528

25 Supplementary figures: 4

26 Supplementary table: 1

27

28

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Highlight *OsANN4* enhances SOD and CAT activities to scavenge H₂O₂ to alleviate the formation of aerenchyma under ABA treatment. OsCDPK24 interacts with and phosphorylates OsANN4, this interaction strengthened under ABA treatment.

Abstract

Plant annexins are calcium- and lipid-binding proteins that have multiple functions, and a significant amount of research on plant annexins has been reported in recent years. However, the functions of annexins in diverse biological processes in rice are largely unclear. Herein, we report that *OsANN4*, a calcium-binding rice annexin protein, is a substrate for OsCDPK24, and the OsANN4 phosphorylation site is the 13th serine, which is a key site for phosphorylation. Most strikingly, abscisic acid (ABA) promotes the interaction between OsANN4 and OsCDPK24. Moreover, knocking down *OsANN4* by RNA interference resulted in visible and invisible phenotypes with exogenous ABA treatment, such as shorter shoots of seedlings, less lateral roots, earlier root aerenchyma formation and so on. The further analyzed results showed that decreased superoxide dismutase (SOD) and catalase (CAT) activity of the RNAi lines, which control H₂O₂ accumulation for redox homeostasis, and further promoted earlier aerenchyma formation of the root. These results suggest that a proposed molecular mechanism exists between OsANN4 and H₂O₂ production to response ABA.

Keywords : Annexin, Calcium-dependent protein kinase, Phosphorylation, H₂O₂, Absciscic acid, *Oryza sativa*

Introduction

Absciscic acid (ABA), a well-known long-distance signaling molecule utilized for communication between plant roots and shoots under water-deficient conditions, is also considered a hormone that plays a critical role in abiotic stress tolerance in plants (Pekic *et al.*, 1995; Cutler *et al.*, 2010). Recently, many mediators involved in ABA signaling, such as ABA receptors (McCourt and Creelman, 2008; Klingler *et al.*,

2010; He *et al.*, 2014) and targets of ABA receptors (Bueso *et al.*, 2014; Ye *et al.*, 2017), have been characterized. Since the identification of the steroidogenic regulatory protein (StAR)-related lipid-transfer (START) domain as a candidate ABA receptor (Ma *et al.*, 2009; Santiago *et al.*, 2009; Nishimura *et al.*, 2010), pyrabactin resistance 1 (PYR1) and PYR1-like 1-13 (PYL1-PYL13) have been considered as key components of the core ABA signaling pathway (Fujii *et al.*, 2009; Ma *et al.*, 2009; Park *et al.*, 2009; Zhao *et al.*, 2013). ABA receptors may inhibit the activity of phosphatase 2Cs (PP2Cs) and result in the activation of Suc nonfermenting-1-related protein kinase 2 (SnRK2s), further promoting the expression of some downstream transcription factors or membrane ion channels (Melcher *et al.*, 2010; Ng *et al.*, 2014).

Exposure to abiotic stresses, such as salt, drought and oxidative stress, adversely affects plant growth and crop productivity. ABA functions as an important phytohormone that has long been known to play a critical role in stress responses (Giraudat *et al.*, 1994; Himmelbach *et al.*, 2003) to regulate the expression of many genes, leading to complex physiological and metabolic responses that enable plants to confer tolerance to abiotic stress (Cutler *et al.*, 2010; Umezawa *et al.*, 2010). Increasing evidence shows that ABA-enhanced abiotic stress tolerance might be associated with the induction of antioxidant defense systems, including reactive oxygen species (ROS) scavenging enzymes, such as catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and non-enzymatic antioxidants (Jiang and Zhang, 2002; Miao *et al.*, 2006; Neill *et al.*, 2008; Xing *et al.*, 2008; Miller *et al.*, 2010; Ozfidan *et al.*, 2012; Ding *et al.*, 2013). Especially the recent study shows in the presence of ABA, the increased production of H₂O₂ activates a calcium-dependent protein kinase (Ni *et al.*, 2018), it suggests ABA induced H₂O₂ production to play a key role in response to abiotic stress.

Annexins belong to an evolutionarily conserved multi-gene protein superfamily comprising Ca²⁺-dependent phospholipid-binding proteins. Calcium ions bind to annexin molecules mainly via coordination sites termed type II (“AB”) high-affinity Ca²⁺-binding sites. In addition, annexins may contain two other binding sites with lower affinity, named the type III “B” and “DE” sites (Moss and Morgan, 2004).

89 Some annexins have no type II Ca^{2+} -binding sites, including some rice annexins,
90 indicating that different protein conformations and different mechanisms for binding
91 phospholipids may exist.

92 Plant annexins are reportedly tissue-specific, and their expression is regulated
93 developmentally. Recent results also suggested that annexins play an important role in
94 plant stress responses (Clark *et al.*, 1998; Lee *et al.*, 2004; Clark *et al.*, 2012; Jami *et*
95 *al.*, 2012; Richards *et al.*, 2014; Qiao *et al.*, 2015), and several plant annexins have
96 been demonstrated to play specific roles in ABA treatment and osmotic stress. The
97 alfalfa (*Medicago sativa*) annexin gene *AnnMs2* was first reported to be activated by
98 drought (Kovacs *et al.*, 1998). *AtANN1* and *AtANN4* play important roles in osmotic
99 stress and ABA signaling in a Ca^{2+} -dependent manner (Jami *et al.*, 2012). *AtANN1*
100 was proven to act as an H_2O_2 sensor (Laohavisit *et al.*, 2010). The expression of
101 annexin upon ROS regulation have been identified in several organism types,
102 including plants and animals (Richards *et al.*, 2014). The rice *OsANN1* gene shows
103 the ability to confer heat and drought stress tolerance by modulating antioxidant
104 accumulation under abiotic stresses (Qiao *et al.*, 2015).

105 Sequence analysis revealed that annexin may have a post-transcriptional
106 modification site such as a phosphorylation site, which may be a substrate for protein
107 kinases (Jami *et al.*, 2012). Evidence obtained using a tandem affinity purification
108 approach to identify protein complexes suggests that annexin may interact with
109 various kinases, including receptor-like kinase, sterile-20 (Ste20)-like kinase,
110 calcium/calmodulin-dependent protein kinase and casein kinase (Rohila *et al.*, 2006).
111 In addition to the MAPK cascade, Ca^{2+} signaling is another critical pathway triggered
112 by environmental stimuli and developmental cues. Calcium-dependent protein kinases
113 (CDPKs) are serine/threonine protein kinases that function as one of the best
114 characterized Ca^{2+} sensors in plants. Previous studies suggested that CDPKs are
115 involved in the responses of plants to various abiotic stresses, including salt, drought,
116 hormonal stimuli and oxidative stress (Kobayashi *et al.*, 2007; Zhu *et al.*, 2007; Asano
117 *et al.*, 2012; Rahoui *et al.*, 2017). *OsCPK24* (same as *OsCDPK24*), the rice CDPK
118 gene, increases the tolerance of rice seedlings by reducing the thioltransferase activity

119 of OsGrx10 to increase proline and glutathione contents under cold stress (Liu *et al.*,
120 2018). The transcription factor protein OsDi19-4 interacts with OsCDPK14/OsCPK24
121 and is further phosphorylated by OsCDPK14/OsCPK24, acting in response to ABA
122 signaling (Wang *et al.*, 2016). However, the interactions between protein kinases
123 CDPKs and annexins that are involved in the regulation of antioxidant defenses in
124 ABA signaling remain to be determined.

125 The aerenchyma is a normal morphological structure utilized by plants to adapt to
126 the waterlogging and submergence of soil, promoting internal oxygen diffusion
127 (Colmer, 2003; Licausi *et al.*, 2011; Licausi, 2013). Ultra structural results suggest
128 that cortical cell death occurs constitutively (Joshi and Kumar, 2012) via a process
129 termed constitutive aerenchyma formation. Under oxygen-deficient conditions, many
130 plant roots will induce aerenchyma development to ensure internal oxygen transport
131 from the shoots to roots, thus facilitating gas diffusion (Campbell and Drew, 1983;
132 Colmer, 2003). Aerenchyma formation is regulated through reactive oxygen species
133 (ROS) in plants (Colmer and Pedersen, 2008; Rajhi *et al.*, 2011; Yamauchi *et al.*,
134 2014). Hydrogen peroxide (H₂O₂), a type of ROS, plays a key role in programmed
135 cell death and lysigenous aerenchyma formation (Yamauchi *et al.*, 2017).

136 In this study, we identified a putative annexin family gene in rice, *OsANN4*. We
137 investigated that *OsANN4* alleviates ABA-induced root aerenchyma formation via
138 modulating H₂O₂ accumulation. Moreover, *OsANN4*, a calcium-binding annexin
139 protein, is an interacting protein of kinase OsCDPK24 and further be phosphorylated
140 by OsCDPK24, and responses to ABA treatment. Thus, we have discovered a
141 proposed mechanism for annexin and protein kinase in H₂O₂ production in
142 aerenchyma formation to response ABA.

143

144 **Materials and methods**

145 *Vector construction for recombinant protein expression and rice transformation*

146 Total RNA was extracted from 7-day-old seedlings of WT plants. Full-length
147 *OsANN4* cDNA without the stop codon was amplified with primers P1 and P2 via

reverse transcription PCR. For sequencing and subcloning, the products were initially ligated into a PMD18-T vector. After sequencing, the fragment was cut with *Xba*I and *Kpn*I and inserted into the PMDC83 expression vector driven by a CaMV35S promoter. To construct the RNA interference vector, the 347-bp coding sequence of *OsANN4* was amplified with primers P3 and P4, digested with *Sac*I and *Spe*I followed by *Bam*HI and *Kpn*I, and subsequently ligated into the pTCK303 vector.

For the Ca^{2+} -binding assay, the expression vector pET28a-*OsANN4* was constructed. Primers P5 and P6 were designed, and the full-length cDNA encoding *OsANN4* was subcloned into *E. coli* between the *Eco*RI and *Hind*III sites in an orientation such that the cDNA sequence was followed by a His tag.

For the pull-down assay, the expression vector pGEX4T-1-*OsANN4* was constructed. The full-length cDNA encoding *OsANN4* was ligated into pGEX4T-1 using primers P5 and P6 to obtain the cDNA sequence followed by a GST tag. The expression vector pET28a-*OsCDPK24* was constructed by using primers P7 and P8.

For the LCI assay, the full-length cDNA encoding *OsANN4* was ligated into pCAMBIA-Cluc by using primers P9 and P10, and *OsCDPK24* was ligated into pCAMBIA-Nluc by using primers P11 and P12.

For the construction of pET28a-*OsANN4*(S13A), the primers P5 and P6 were used and the vector of *OsANN4*(S13A)-Cluc was constructed by using primers P9 and P10. Site-directed mutagenesis was finished by a fast mutagenesis kit (Tiagen, China).

All primer sequences used in these experiments are listed in Table S1. The expression vectors pET28a-*OsANN4* and pGEX4T-1-*OsANN4* were then transformed into the *E. coli* Rosetta strain. In addition, other constructs were introduced into *Agrobacterium* EHA105 and transformed into rice calli. Transgenic rice plants were generated as previously described.

Plant materials and stress treatment

The rice (*Oryza sativa* subsp. japonica) cultivar Nipponbare was used in this study and as the WT control in all experiments. Rice seeds were surface-sterilized with 50%

NaClO for 20 min, rinsed 10 times with sterile distilled water and then grown on 1/2 MS medium. The rice plants were grown in a standard culture solution in a greenhouse with a light/dark cycle of 16/8 h and 50% relative humidity at 28/25 °C (day/night). For the ABA sensitivity assay, the seeds were planted on 1/2 MS medium supplemented with 10 µM ABA for 12 days. Control seeds were planted on 1/2 MS medium and cultured with water after 12 days.

183

184 *RNA isolation, RT-PCR, and quantitative RT-PCR analysis*

Total RNA from different tissues of rice plants was isolated using the TaKaRa RNAiso Plus kit. Purified RNA (2 µg) was incubated with DNase 1 (RNase-Free DNase, Thermo Fisher, USA) at 37 °C for 30 min. First-strand cDNA was synthesized using the PrimeScript™ First Strand cDNA Synthesis Kit (TaKaRa, Japan) to perform RT-PCR. For qRT-PCR, 1µg purified total RNA was used to obtain the first-strand cDNA with the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Japan).

The Takara SYBR® Premix Ex Taq™ II was used to perform the qRT-PCR assay using primers P13 and P14. The C1000 Real-Time PCR instrument (Bio-Rad, USA) was used for qRT-PCR. *OsACTIN1* (Os03g0718100) was used as an internal control for the normalization of all data by using Primers P15 and P16 in this experiment. Three independent biological replicates were assayed.

196

197 *Sectioning and microscopy of root tips*

Adventitious roots were obtained from 1/2 MS medium supplemented with 10 µM ABA. After embedding in resin (SPI, USA), the embedded materials were measured and marked at 3 mm and 5 mm from the root tip. Then the transverse sections of 1 µm thickness were acquired on a RM 2265 rotary microtome (Leica, Germany). And the resin section was stained with toluidine blue, and examined and photoed by a DVM6a 3D microscope (Leica, Germany).

204

205 *Measurement of H₂O₂ content*

206 H_2O_2 content in rice seedlings was determined using the Hydrogen Peroxide Assay
207 Kit (Beyotime, China). According to the instructions, the samples were fully
208 homogenized in lysis buffer. Fifty microliters of supernatant was added to 100 μ l of
209 test solution and then placed at 30 °C for 30 min. Readings at a wavelength of 560 nm
210 were measured immediately. Then, H_2O_2 content was calculated according to the
211 standard curve.

212

213 *Superoxide dismutase and catalase activity assays*

214 To determine the total soluble protein content, rice seedlings (0.2 g, fresh weight,
215 FW) were homogenized in 1.0 ml of extraction buffer (50 mM phosphate, 1% (w/v)
216 polyvinyl pyrrolidone, pH 7.8) in a mortar on ice. The plant tissue was ground into a
217 homogenate and then centrifuged at 4 °C for 20 min at 8000 rpm. The supernatants
218 were used for assaying the protein content and enzyme activities. The total soluble
219 protein contents were then determined by the Bradford method using bovine serum
220 albumin (BSA, Solarbio, China) as the standard. All spectrophotometric analyses
221 were performed on a METASH UV-5200PC spectrophotometer (Shanghai, China).

222

223 *Assaying the in situ localization of H_2O_2*

224 DAB staining was performed following a published method with some
225 modifications. 7-day-old rice seedlings were treated with 10 μ M ABA, and then the
226 leaves were detached and immersed in a solution containing 1 mg/ml DAB prepared
227 in HCl-acidified (pH 3.8) water at 25 °C for 10 h and the roots were immersed in
228 DAB solution for 4h. The samples were then incubated in a boiling solution (60%
229 ethanol, 20% glycerol and 20% ethylic acid) to wash off green color and then
230 observed and imaged under a DVM6a 3D microscope (Leica, Germany).

231

232 *Yeast two-hybrid analysis*

233 The *OsANN4* coding sequences were cloned into the BD (pGBKT7) vector

234 between the *Nde*I and *Eco*RI sites with primers P17 and P18. The *OsCDPK24*
235 (Os11g0171500) coding sequence was cloned into AD (pGADT7) vectors by using
236 primers P19 and P20. The expression vectors pGADT7-*OsCDPK24* and
237 pGBKT7-*OsANN4* were co-transformed into the yeast strain AH109 via the lithium
238 acetate method (Clontech Yeast Protocols and book). Colonies were then transferred
239 to SM-LWHA and SM-LW. A positive control interaction between the 53 protein and
240 SV40 protein and a negative control interaction between the Lam protein and SV40
241 protein were observed.

242

243 *Pull down assays*

244 For induction and purification of *OsCDPK24*, the plasmid pET28a-*OsCDPK24* was
245 induced at 18°C overnight in *E. coli* strain Rosetta and purified with Ni-NTA Resin.
246 *OsANN4*-GST and GST were induced at 18°C overnight in *E. coli* strain Rosetta and
247 purified with glutathione Sepharose 4B beads (GE). Supernatant containing GST or
248 *OsANN4*-GST was incubated with *OsCDPK24*-His in 0.5 ml of interaction buffer (25
249 mM Tris pH 7.2, 150 mM NaCl) overnight at 4°C. GST beads were added and
250 incubated with proteins for 2 h on a rotating wheel at 4°C followed by washing five
251 times with wash buffer (25 mM Tris pH 7.2, 150 mM NaCl, 0.1% NP-40). Then, the
252 samples were boiled with 120 µl of 1× SDS loading buffer at 100°C for subsequent
253 western blot analysis.

254

255 *Luciferase complementation imaging (LCI) assay*

256 *OsANN4* and *OsCDPK24* were constructed by using pCAMBIA-Cluc and
257 pCAMBIA-Nluc, respectively, and were transformed into *N. benthamiana* leaves by
258 *Agrobacterium* strain GV3101. *Agrobacterium* transformants containing
259 p*OsANN4*-Cluc, p*OsCDPK24*-Nluc, empty control pCAMBIA-Cluc and
260 pCAMBIA-Nluc were adjusted to OD₆₀₀=0.5-0.6 and then injected into tobacco
261 leaves. After spraying the tobacco leaves with 2.5 mM D-luciferin (Goldbio, USA),
262 fluorescent signals were detected and photographed post infiltration by using a Fusion
263 FX7 (Vilber, France) imaging system. The cold luminescence signals values of

264 OsANN4 and OsCDPK24 were calculated by Fusion FX7 (Vilber, France).

265

266 *In vitro kinase assays*

267 Briefly, 5 µg of OsANN4-His was incubated with 1.5 µg of protein kinase
268 OsCDPK24-His in kinase buffer (20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 100 mM
269 NaCl, 1 mM DTT), and 2 mM ATP was added. The reactions were incubated at 37 °C
270 for 10 h and stopped by boiling with 6× SDS loading buffer. After adding 50 mM
271 Phos-Tag (APExbio, China) and 100 mM MnCl₂, the samples were separated by
272 SDS-PAGE and then transferred to nitrocellulose membranes. The signals were
273 detected with a His-tag mouse monoclonal antibody (CWbio, China). Mass
274 spectrometry to detect phosphorylation was finished by Applied Protein Technology
275 Co. Ltd company (Shanghai, China).

276

277 *Fluorescence measurements of OsANN4*

278 This experiment was finished according to the method described in the study
279 previously (Zhou *et al.*, 2011). 2 mM Ca²⁺ was added into 2 µM recombinant protein,
280 and the fluorescence signals was measured by fluorescence spectrophotometer
281 (F-4600; Hitachi, Japan).

282

283 *Subcellular localization of OsANN4*

284 To examine the subcellular distribution of OsANN4, the 35S::OsANN4-GFP vector
285 was constructed. The OsANN4 coding region was fused to GFP using the PMDC83
286 backbone to construct CaMV35S::OsANN4-GFP, and its expression was driven by
287 the 35S promoter. The constructs were introduced into *Agrobacterium* EHA105 and
288 then transformed into rice calli. Transgenic rice plants expressing OsANN4-GFP were
289 generated as previously described. The signal of OsANN4-GFP protein was examined
290 using confocal laser-scanning microscopy (Zeiss LSM710, Germany).

291

292 **RESULTS**

293 *OsANN4* responds to exogenous abscisic acid in rice

294 We cloned and obtained a putative annexin family gene in rice, *OsANN4*, which is a
 295 ortholog of *AtANN4* (Fig. 1A). The genomic sequence of *OsANN4* consists of 5 exons
 296 and 4 introns, and the open reading frame is predicted to encode a protein with 320
 297 amino acids. Based on sequence searches of the NCBI database, the *OsANN4* protein
 298 contains three annexin domain architectures, the so-called annexin repeats, which
 299 comprise segments of 32, 57, and 67 amino acid residues. Analysis of the 1794-bp
 300 promoter sequence upstream of *OsANN4* reveals that the *OsANN4* promoter contains
 301 multiple cis-acting elements that may be associated with abiotic stress (Fig. S1). Then
 302 we treated 7-d-old seedlings with 10 μ M ABA and detected the expression of
 303 *OsANN4* at 0 h, 0.5 h, 1 h, 3 h, 6 h, 12 h and 24 h. The results showed that ABA
 304 treatment induced the expression of *OsANN4* and the highest expression level after
 305 ABA treatment for 6 h, which showed that *OsANN4* may respond to exogenous
 306 abscisic acid (Fig. 1B).

307 To test whether *OsANN4* is sensitive to ABA treatment, fifteen independent
 308 knocking down *OsANN4* transgenic lines were generated. Quantitative real-time PCR
 309 analyses showed that *OsANN4* was down regulated in all the RNAi lines (Fig. 1D).
 310 The homozygous plants of R4, R12 and R15 showed obviously decreased
 311 transcriptional level of *OsANN4* and to be used for the further analyzed. Seeds from a
 312 wild-type (WT) line and three RNAi lines (R4, R12 and R15) were planted on 1/2
 313 Murashige and Skoog (MS) medium supplemented with 10 μ M ABA, and on 1/2 MS
 314 medium as a control. Twelve days later, both WT and RNAi plants showed no
 315 significant difference at the stage of early seedling growth without ABA treatment
 316 (Supplementary Fig. S2A). Otherwise, the shoots of RNAi lines were shorter than WT
 317 with ABA (Fig. 1C, E). Furthermore, the length of the root and the numbers of
 318 adventitious roots were not significantly different from that of the wild type after
 319 ABA treatment, whereas the number of lateral roots was significantly less relative to
 320 wild type line (Fig. 1E, F). This result indicates that the RNAi seedlings are sensitive
 321 to ABA in the early seedling stage.

To understand if *OsANN4* expression is involved in ABA response, we further examined the expression levels of *OsANN4* and several genes related ABA biosynthesis and response in RNAi lines and WT treated with 10 μ M ABA for 0h, 0.5h, 1h, 3h and 6h. The expression levels of *OsANN4* in RNAi lines were always lower relative to WT (Supplementary Fig. S2B). Under both normal and ABA treatment, *OsNCED4* and *RABI6A* expression were decreased in the RNAi lines, especially in 6h (Supplementary Fig. S2C, S2E). However, the expression of the *OsZEP* transcripts did not differ between the WT and RNAi lines (Supplementary Fig. S2D). These data sufficiently demonstrated that *OsANN4* is closely related to ABA response.

332

333 *OsANN4* modulates ROS in response to ABA in rice roots

More evidence suggests that ABA-enhanced stress tolerance is associated with the induction of the antioxidant defense system to protect plant cells against oxidative damage (Xing *et al.*, 2008; Ozfidan *et al.*, 2012). SOD and CAT may protect plant cells from oxidative damage by eliminating ROS, which are important signaling molecules in the ABA pathway. SOD is a critical enzyme that catalyzes O_2^- to H_2O_2 , and CAT is a key enzyme that catalyzes H_2O_2 to H_2O and O_2 which protects plant cells from oxidative damage by eliminating ROS. To assess the effect of knocking down *OsANN4* on antioxidant defense to ABA response, we detected the activities of SOD and CAT in 7-d-old seedlings without or with 10 μ M ABA treatment for 1 h. The RNAi lines showed no significant difference in activities of both SOD and CAT relative to those of the WT without treatment. After ABA treatment for 1h, both the SOD and CAT activities of RNAi lines were decreased relative to WT (Fig. 2A, B).

We further detected H_2O_2 content in 7-d-old RNAi and WT seedlings. The RNAi lines showed increased H_2O_2 content in both shoots and roots after 10 μ M ABA treatment (Fig. 2C, D). The results were consistent with SOD and CAT activity. Next, we assayed the production of H_2O_2 in situ of RNAi lines and WT. Seven-day-old rice seedlings were treated with or without 10 μ M ABA for 5h, roots and leaves were then

351 subjected to 3, 3'-diaminobenzidine (DAB) staining respectively. Without ABA
352 treatment, there were no visible differences in rice roots and leaves of WT and RNAi
353 lines. However, increased numbers of darkly stained patches were observed in
354 mesophyll cells and root cells of RNAi lines (Fig. 2E, F), indicating an increased level
355 of H₂O₂ in these plants.

356

357 *OsANN4 promotes stomatal closure and alleviates H₂O₂-induced aerenchyma*
358 *formation with ABA treatment*

359 It is reported that a certain ROS content resulted in programmed cell death and
360 further promoted aerenchyma formation (Yamauchi *et al.*, 2017). The H₂O₂ content
361 was obviously increased in the roots of the *OsANN4*-RNAi lines, then we investigate
362 whether *OsANN4* affects aerenchyma formation in adventitious roots. Twelve-day-old
363 *OsANN4*-RNAi lines as well as WT were planted in 1/2 MS medium supplemented
364 with 10 μM ABA. Upon analysis of the transverse sections, aerenchyma formation
365 was observed only in the RNAi lines and not in the WT at 3-mm from root tips.
366 Furthermore, aerenchyma formation was observed in both RNAi lines and WT at the
367 5-mm transverse sections (Fig. 3A, B). Then we also detected H₂O₂ content of
368 12-d-old RNAi and WT seedlings with 10 μM ABA treatment. The RNAi lines
369 showed higher H₂O₂ content relative to WT with ABA treatment, which may result in
370 an increase in the number of aerenchyma in the RNAi lines (Supplementary Fig. S3).

371 We further assessed the stomatal apertures of these plants using scanning electron
372 microscopy images of rice leaves. The proportion of stomatal closure in WT was
373 significantly increased with ABA treatment than that without ABA treatment.
374 However, for RNAi plants, the statistical showed no significantly differences of
375 stomatal openness and closure with or without 10 μM ABA treatment (Fig. 3C, D). It
376 suggests that *OsANN4* may play a key role in ABA-induced stomatal closure.

377

378 *OsANN4 interacts with the protein kinase OsCDPK24*

379 In previous reports, annexins were shown to interact with protein kinases, including
380 SAPKs and CDPKs (Rohila *et al.*, 2006; Qiao *et al.*, 2015). To further understand how

381 *OsANN4* response to ABA, we used yeast two-hybrid assay to verify several potential
382 rice protein kinase candidates, including Os01g0570500, Os10g0518800,
383 Os01g0869900, Os11g0171500. With the results, we did not find Os01g0570500 and
384 Os10g0518800 interacted with *OsANN4* separately. However, Os01g0869900,
385 belongs to sucrose nonfermenting1-related protein kinase2 (SnRK2) family, showed
386 weak interaction with *OsANN4*. Furthermore, *OsCDPK24* (Os11g0171500), a key
387 regulator in response to ABA (Wang *et al.*, 2016), showed strong interaction with
388 *OsANN4* (Fig. 4A).

389 To further verify the interactions between *OsANN4* and *OsCDPK24*, the pull-down
390 system in vitro was carried out. *OsCDPK24*-His and *OsANN4*-GST recombinant
391 proteins were induced in *Escherichia coli* and purified to perform the pull-down assay.
392 *OsANN4*-GST could pull down *OsCDPK24*-His, whereas GST could not, it was
393 further proved the interaction between *OsANN4* and *OsCDPK24* (Fig. 4B).

394 We also obtained additional confirmation of the interaction between *OsANN4* and
395 *OsCDPK24* in *Nicotiana benthamiana* leaves by using a luciferase
396 complementation imaging (LCI) assay. After spraying D-luciferin to tobacco leaves, a
397 fluorescent signal occurred when p*OsANN4*-Cluc and p*OsCDPK24*-Nluc were present
398 simultaneously, which showed that *OsANN4* can interact with *OsCDPK24* (Fig. 4C).

399

400 *The 13th serine residue is a key phosphorylation site for OsANN4*

401 To understand the mechanism underlying the interaction of *OsANN4* and
402 *OsCDPK24*, we performed a phosphorylation assay in vitro to determine whether
403 *OsANN4* is a substrate of *OsCDPK24*. The Phos-tag reagent was used to separate
404 phosphorylated proteins from non-phosphorylated proteins according to their different
405 migration rates. When purified *OsANN4*-His and *OsCDPK24*-His were incubated
406 together, phosphorylated *OsANN4* bands were detectable with a His-tag antibody.
407 The phosphorylation level of *OsANN4* increased slightly after the addition of 5 μ M
408 and 500 μ M Ca^{2+} . These results indicated that *OsANN4* can be phosphorylated by
409 *OsCDPK24*, especially in the presence of Ca^{2+} (Fig. 5A).

410 To further demonstrate that *OsANN4* was a substrate of *OsCDPK24*, mass

spectrometry was performed to detect specific phosphorylation sites of OsANN4 when OsCDPK24 was present. Mass spectrometry analysis showed that OsANN4 can be phosphorylated by OsCDPK24, and the OsANN4 phosphorylation site was the 13th amino acid, which is a serine (Fig. 5B).

The interaction of OsANN4 and OsCDPK24 plays a role in the response to ABA

To determine whether the interaction of OsANN4 and OsCDPK24 plays a role in the response to ABA, we performed 10 μ M ABA treatment on tobacco leaves injected with *Agrobacterium* GV3101 containing pOsANN4-Cluc and pOsCDPK24-Nluc. After ABA treatment, LUC cold luminescence signals increased significantly, which demonstrated the interaction between OsANN4 and OsCDPK24 was enhanced with ABA treatment (Fig. 6A and B). These results indicated that OsANN4 interacted with OsCDPK24, and this interaction increased under ABA treatment, thus, the OsANN4-OsCPK24 interaction may play a role in the response to ABA in rice.

To demonstrate whether the phosphorylation of OsANN4 plays a role in response to ABA signaling, we mutated the serine (S) residue with non-phosphorylatable alanine (A) to inhibit the phosphorylation of OsANN4 and constructed the pOsANN4 (S13A)-Cluc vector to perform an LCI assay. We found that after mutation of phosphorylation site in OsANN4, OsANN4 still can interact with OsCDPK24. After spraying with ABA, the responses of OsANN4 (S13A)-Cluc and OsCDPK24-Nluc to ABA were significantly weakened (Fig. 6C, D). This suggests that this key phosphorylation site plays an important role in *OsANN4* response to ABA.

OsANN4 is a Ca^{2+} -binding protein that locates to the cell periphery

Previous studies have shown that the subcellular localization of annexin may be altered due to environmental stimuli (Baucher *et al.*, 2012; Qiao *et al.*, 2015). To explore whether OsANN4 altered its subcellular localization with ABA treatment, 35Spro::OsANN4-GFP was introduced into *Agrobacterium* EHA105 and then transformed the rice callus. Twenty-five independent transgenic lines were obtained, and 3 lines were used for further analyses. We observed OsANN4 was located to the

cell periphery by a confocal laser-scanning microscope (Zeiss LSM710). After 10 μ M ABA treatment, the signal of OsANN4-GFP remained unchanged (Supplementary Fig. S4).

Annexins are considered to be a class of protein interacting with biological membranes in a calcium dependent or independent manner. In this study, we detected the Ca^{2+} binding activity of OsANN4. The fluorescence level of OsANN4-His recombinant protein was determined by fluorescence spectrophotometer (F-4600; Hitachi, Japan). Upon excitation at 315 nm (Fig. 5C), the fluorescence emission spectrum showed the maximum fluorescence wavelength (λ max) at 390 nm, where the fluorescence intensity reached approximately 2000 A.U.. The maximum fluorescence intensity of the OsANN4-His recombinant protein was measured again after the addition of 2 mM Ca^{2+} . The results showed that the maximum fluorescence wavelength remained unchanged, whereas the fluorescence intensity changed (Fig. 5E), indicating that the OsANN4 has Ca^{2+} binding activity and may further change the conformation of the protein.

Since the phosphorylation site of OsANN4 plays a role in the ABA response, we examined whether this phosphorylation site affects its binding to Ca^{2+} . OsANN4 (S13A)-His recombinant protein was induced and purified to perform the above fluorescence assay. The results showed that upon excitation at 315 nm (Fig. 5D), the fluorescence emission spectrum of OsANN4 (S13A)-His recombinant protein remained unchanged whereas the fluorescence level has changed (Fig. 5F), which indicated that the phosphorylation site of OsANN4 does not affect its binding to Ca^{2+} but may change the conformation of OsANN4.

Discussion

OsANN4 modulates ROS production to alleviate aerenchyma formation

Recent results have revealed that annexins are involved in the responses to various abiotic stresses including salt, drought, oxidative and other stresses (Clark *et al.*, 1998; Lee *et al.*, 2004; Clark *et al.*, 2012; Jami *et al.*, 2012; Richards *et al.*, 2014; Qiao *et al.*, 2015). Evidence has shown that induction of the antioxidant defense system protects

plant cells against oxidative damage (Mittler *et al.*, 2011; Richards *et al.*, 2014; Del Rio, 2015; Qiao *et al.*, 2015). And ROS are important signaling molecules, especially in the ABA signaling pathway. Herein, we reported *OsANN4*, a ortholog of *AtANN4*, modulates H₂O₂ accumulation to alleviate root aerenchyma formation with exogenous abscisic acid. Our results showed that knocking down *OsANN4* decreased the activity of SOD and CAT with ABA treatment, and more H₂O₂ content was measured (Fig. 2), further resulted in earlier aerenchyma formation (Fig. 3A, B). We conclude that *OsANN4* promotes redox reactions, potentially regulating SOD and CAT to scavenge H₂O₂.

Hydrogen peroxide (H₂O₂), is also an essential secondary messenger to be involved in ABA-induced stomatal closure. Interestingly, in our study, a high level of H₂O₂ content did not result in a high proportion of stomatal closure in *OsANN4*-RNAi lines (Fig. 3D). Abscisic acid causes the Ca²⁺ concentration in cytoplasmic to elevate in guard cells via Ca²⁺ influx, results in stomatal closure (Hedrich, 2016; Kong *et al.*, 2016). We speculate that *OsANN4*, a putative Ca²⁺ channel, may function in stomatal closure by affecting Ca²⁺ influx under ABA treatment.

OsANN4 mediates Ca²⁺ and interacts with OsCDPK24 in response to ABA

In rice, a previous study showed that a rice calcium-dependent protein kinase *OsCDPK12* can induce expression of the antioxidant genes *OsAPX2* and *OsAPX8* under salt stress and reduce the salt-induced accumulation of H₂O₂ (Asano *et al.*, 2012), suggesting that *OsCDPK12* positively regulates ROS detoxification by controlling the expression of antioxidant genes. *ZmCPK11* has been proven to be involved in ABA-induced antioxidant defense and to function upstream of *ZmMPK5* in ABA signaling in maize (Ma *et al.*, 2016). However, the regulatory network regulating antioxidant defense in ABA signaling remains to be elucidated. In this study, we demonstrated that *OsANN4* interacts with the kinase *OsCDPK24* (Fig.4), and furthermore, this interaction plays a role in the ABA response (Fig. 6). Moreover, *OsANN4* is a substrate for *OsCDPK24* and *OsANN4* phosphorylation site is the 13th serine (Fig. 5), which is a key site for phosphorylation. After mutated the serine

residue with non-phosphorylatable alanine (A) to inhibit the phosphorylation of OsANN4, the conformation of OsANN4 has changed (Fig. 5F) and the interaction of OsANN4 and OsCDPK24 was weakened (Fig. 6C, D), indicating that under ABA treatment, OsCDPK24 may be more inclined to interact with the phosphorylated OsANN4, rather than the non-phosphorylated state. RCAR is a component of the ABA receptor complex, and PP2C phosphatases inhibit the activity of some kinases, similar to OsCDPK, which can be dephosphorylated and promote ion channel expression (Kudla *et al.*, 2018) in response to ABA. However, whether CDPKs are involved in ABA-induced antioxidant defense remains to be determined.

Annexins are traditionally perceived as Ca^{2+} -dependent phospholipid-binding proteins, which usually contain a characteristic type II Ca^{2+} -binding residue in each corresponding repeat in vertebrates. In plant annexins, the type II Ca^{2+} -binding residues are absent in repeats 2 and 3, and no type II Ca^{2+} -binding residues are found in OsANN4 (predicted at ScanProsite <http://ca.expasy.org/tools/scanprosite/>). However, our results indicate that OsANN4 has the Ca^{2+} binding activity, which could be perceived as evidence that annexins can also function in their Ca^{2+} -free conformation in an unknown intricate fashion, thus increasing the functional diversity of annexins. After mutating the phosphorylation site of OsANN4, the protein conformation of OsANN4 is altered, but it still can bind to Ca^{2+} (Fig. 5F). What's more, some data have indicated that ion channels or transporters act as targets of Ca^{2+} signaling networks (Whalley *et al.*, 2011; Edel and Kudla, 2015; Kudla *et al.*, 2018). As a putative ion channel, OsANN4 can interact with OsCDPK24, supporting the existence of potential connection of the annexin, calcium and protein kinase in response to stress.

525

A possible model for OsANN4 to respond to ABA

The conformation of PYL can be changed after binding ABA, thus enhancing the stability of the complexes of PYL and PP2C, resulting in a reduced inhibitory effect of PP2C on kinases (SnRK or CDPK, etc.), which then activates transcription factors, ion channels or other ABA-responsive genes (Finkelstein, 2013; Kudla *et al.*, 2018;

Wang *et al.*, 2018). Based on our experimental results, we proposed a working model for OsANN4 functioning as a key protein in modulating H₂O₂ accumulation to alleviate root aerenchyma formation with exogenous abscisic acid (Fig. 7). OsANN4 is a Ca²⁺-binding protein, and it can interact with and be phosphorylated by OsCDPK24 at the 13th serine. When ABA is present, PP2C binds to the PYL receptor, may result in a decrease in the inhibitory effect of PP2C on OsCDPK24. The interaction between OsANN4 and OsCDPK24 is enhanced to response ABA. When OsCDPK24 interacts with OsANN4, it may be more inclined to phosphorylate OsANN4 under ABA treatment. *OsANN4* promotes O₂⁻ to H₂O₂ by increasing SOD activity, then converts H₂O₂ into H₂O through CAT activity, thereby reducing ROS content (O₂⁻ and H₂O₂). As a consequence, *OsANN4* can further alleviate programmed cell death and inhibit the formation of aerenchyma.

543

544 **Supplementary data**

545 Supplementary data are available at JXB online.

546 Fig. S1. Analysis of cis-acting elements in the promoter of *OsANN4*.

547 Fig. S2. The relative transcript levels of *OsANN4*, *OsZEP*, *OsNCED4* and
548 *OsRAB16A* in WT and *OsANN4*-R plants with ABA treatment

549 Fig. S3. The H₂O₂ content in the roots of 12-d-old RNAi and WT seedlings planted
550 in 1/2 MS medium supplemented with or without 10 μM ABA.

551 Fig. S4. Subcellular localization of OsANN4-GFP in the 3-day-old root tips cell
552 with or without ABA treatmentl.

553 Table S1. Primer sequences used in plasmid construction and qPCR.

554

555 **Acknowledgements**

556 This work was supported by the National Natural Science Foundation of China
557 (31571638, 31340046), Research Fund for the Doctoral Program of Higher Education
558 (20131303110005) and the Advanced Postdoctoral Science Programs Foundation of
559 He'bei Educational Committee (B2016003012).

We thank Dr. Kang Chong of the Institute of Botany, Chinese Academy of Science, for providing the pTCK303 vector. We thank Shanghai Applied Protein Technology Co. Ltd for the technical assistance in LC/MS proteomic assays.

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Fig. 1. *OsANN4* responses to exogenous abscisic acid in rice.

A Phylogenic tree of *OsANN4* and the *Arabidopsis* orthologue AtANNs. **B** *OsANN4* transcript expression levels under ABA treatment. **C** WT and *OsANN4* RNAi lines were grown for 12 d in 10 μ M ABA medium. The figure shows the representative results of five replicates with T2 generation rice. Scale bars are 1 cm. **D** Relative transcript levels of *OsANN4* in *OsANN4* RNAi transgenic rice. **E**. The length of shoots and roots in 12-d-old WT and *OsANN4* RNAi plants with 10 μ M ABA treatment. **F** The numbers of adventitious roots and lateral roots in 12-d-old WT and *OsANN4* RNAi lines with 10 μ M ABA treatment. Values represent the mean $\pm$ SD from three independent experiments. Statistical significance was determined by Student's t-test, *P<0.05, **P<0.01, ***P<0.001.

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765 **Fig. 2.** *OsANN4* modulates ROS in response to ABA in rice roots

766 **A** SOD activities in 7-d-old WT and *OsANN4*-R plants with or without 10 μ M ABA treatment for 1h. **B**

767 CAT activities in 7-d-old WT and *OsANN4*-R plants with or without 10 μ M ABA treatment for 1h. **C**

768 The H_2O_2 content in the shoots of 7-d-old WT and *OsANN4*-R plants with or without 10 μ M ABA

769 treatment for 1h. **D** The H_2O_2 content in the roots of 7-d-old WT and *OsANN4*-R plants with or without

770 10 μ M ABA treatment for 1h. **E** Histochemical detection of H_2O_2 in situ in the leaves of WT and

771 *OsANN4*-R plants with or without ABA treatment. **F** Histochemical detection of H_2O_2 in situ in the

772 roots of WT and *OsANN4*-R plants with or without ABA treatment. Scale bars for images E. and (f) are

773 1 mm. Values represent the mean $\pm$ SD from three independent experiments. Statistical significance

774 was determined by Student's t-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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795 **Fig. 3.** *OsANN4* promotes stomatal closure and alleviates H₂O₂-induced aerenchyma
796 formation with ABA treatment.

797 **A** Scanning electron microscopy images of three levels of stomatal apertures. **B** The percentage of
798 three levels of stomatal apertures in the leaves of *OsANN4* RNAi and WT plants under normal and
799 ABA treatment. Values are means $\pm$ SD (23 $\leq$ n $\leq$ 36). *P<0.05, **P<0.01. **C** Cross sections at 3 mm
800 and 5 mm from the tips of the adventitious roots in WT and *OsANN4*-R plants with 10 μ M ABA
801 treatment for 12 d. Sections were stained with toluidine blue. Scale bars are 20 μ m. **D** The aerenchyma
802 numbers in the adventitious roots of WT and *OsANN4*-R plants with ABA treatment. Values represent
803 the mean $\pm$ SD from three independent experiments. Statistical significance was determined by
804 Student's t-test, *P<0.05, **P<0.01, ***P<0.001.

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825 **Fig. 4.** OsANN4 can interact with the protein kinase OsCDPK24.

826 **A** Yeast two-hybrid system was used to detect the interaction between OsANN4 and OsCDPK24. **B** A

827 GST pull-down assay was used to detect the interaction between OsANN4 and OsCDPK24. **C** Firefly

828 luciferase complementation imaging (LCI) assay detecting the interaction between OsANN4 and

829 OsCDPK24. The colored scale bar indicates the luminescence intensity.

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855 **Fig. 5.** OsANN4 is a Ca^{2+} -binding protein that can be phosphorylated by OsCDPK24

856 **A** An in vitro kinase assay was performed with purified OsANN4-His and OsCDPK24-His using a

857 Phos-tag gel. Signals were detected by using a His-tag antibody. **B** Mass spectrometry analysis of

858 OsANN4 phosphorylation. **C** The fluorescence excitation of the purified OsANN4-His protein. **D** The

859 fluorescence excitation of the purified OsANN4(S13A)-His protein. **E** The emission spectra of the

860 purified OsANN4-His protein. **F** The emission spectra of the purified OsANN4(S13A)-His protein.

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Fig. 6. The interaction between OsANN4 and OsCDPK24 involved in response to ABA signals

A Firefly luciferase (LUC) complementation imaging(LCI) assay detecting the interaction between OsANN4 and OsCDPK24 with or without ABA treatment. The colored scale bar indicates the luminescence intensity. **B** Quantification of the relative cold luminescence signals displayed in the two group of A.. Values represent mean $\pm$ SD and the single asterisk represent a significant difference determined by the Student's test, * $P < 0.05$, $n > 13$. **C** Firefly luciferase complementation imaging (LCI) assay detecting the interaction between OsANN4-(S13A) and OsCDPK24 with ABA treatment. The colored scale bar indicates the luminescence intensity. **D** Quantification of the relative cold luminescence signals displayed between OsANN4-(S13A) and OsCDPK24 with ABA treatment. Values represent the mean $\pm$ SD and statistical significance was determined by Student's t-test, *** $P < 0.001$, $n > 12$.

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Fig. 7. A proposed model for OsANN4 in response to ABA

The conformation of PYL can be changed after binding ABA, thus enhancing the stability of the complexes of PYL and PP2C, resulting in reducing the inhibitory effect of PP2C on OsCDPK24. OsCDPK24 interacts with OsANN4 and further phosphorylates OsANN4. OsANN4 enhances SOD and CAT activities to scavenge H_2O_2 . As a consequence, OsANN4 can further alleviate the formation of aerenchyma. Under ABA treatment, the interaction between OsANN4 and OsCDPK24 is strengthened, and OsANN4 may be more inclined to be phosphorylated by OsCDPK24.

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A **C** **B** **D**

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OsCDPK24-His	+	+	+	+	+	+	+
OsANN4-His	-	-	+	+	+	+	+
His	-	+	-	-	-	-	-
5 μ M Ca^{2+}	-	-	-	-	-	+	-
500 μ M Ca^{2+}	-	-	-	-	-	-	+
0.2 mM ATP	+	+	+	-	+	+	+

B

R20180500554_175, Cmpd 26209, +MSn(1035.5035), 53.23 min Seq: AFSGLGLGVDEPAMVSALAK Mod: 3,Phospho[S]