OsANN4 confers stress induced by ABA signaling and regulates antioxidant accumulation

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Abstract: Annexins are Ca2+-dependent phospholipids-binding proteins constituting evolutionary conserved multigene family that widely distributed throughout animals and plants. OsANN4 is a member of rice annexin protein family. However, the functions and mechanisms of OsANN4 in stress responses and signal pathways remain unclear. GUS staining assay and quantitative RT-PCR indicated that the expression of OsANN4 is tissue-specific. In this study, OsANN4 expression was high at seedling stage. The plants which knocking down OsANN4 through RNA interference were more sensitive to ABA at the seeds germination stage comparing to wild type. It suggested that OsANN4 was involved in response to ABA signaling under abiotic stress. Furthermore, yeast two-hybrid assays demonstrated that OsANN4 interacted with OsCDPK24. This may provide potential regulation cross-talk in the abiotic stress response.

Key words: Molecular biology; annexin; calcium binding activity; ABA signaling; Oryza Sativa

0　Introduction

Annexins are an evolutionarily conserved group of multigene protein superfamily with Ca^{2+} dependent phospholipids-binding proteins. Ca^{2+} bind to annexin molecule mainly up to the possible coordination site the so-called type II ("AB") high-affinity Ca^{2+}-binding site. In addition, annexins may contain two other binding sites with lower affinity, named type III ("B", "DE") site. In vertebrates, most annexin repeats contain at least one type II Ca^{2+}-binding site individually. In plants, the type II Ca^{2+}-binding residues generally exist in repeats 1 and 4, while often lost in repeats 2 and 3[1,2] . Some even with no type II Ca^{2+}-binding sites, including some rice annexins, indicating that there may exist different protein conformations and different mechanisms for binding phospholipids. There are conserved residues that are important for peroxidase and ATPase/GTPase activity[3].

Plant annexins are reported to be tissue-specific and their expression is regulated developmentally. Recent results also suggested that annexins played an important role in plant stess responses [4-11]. Several plant annexins have been implicated a role specifically in ABA treatment and osmotic stress. Alfalfa (*Medidago sativa*) annxin gene *AnnMs2* was first reported that it was activated by drought, osmotic stress and ABA[12]. *AtANN1* and *AtANN4* play important roles in osmotic stress and ABA signaling in a Ca^{2+} dependent manner[7]. *AtANN1* has been proposed to act as a H_2O_2 sensor [13]. Annexin genes are up-regulated by ROS. The cotton *GhANN1* gene is not only a potential antioxidant role [14], but also is involved in drought and salt stress tolerance[15]. The rice *OsANN1* we've studied recently shows the ability to confer heat and drought stress tolerance by modulating antioxidant accumulation under abiotic stresses [11].

Exposure to abiotic stresses such as salt, drought and oxidative stresses adversely affects the growth of plants and productivity of crops. Abscisic acid is an important phytohormone has long been known to play a critical role in stress responses [16-17], which accumulating under water stress, regulates the expression of many genes and leads to complex physiological and metabolic response enabling plants to confer tolerance to abiotic stresses [18-19]. More and more evidences showed that ABA-enhanced water stress tolerance might be associated with the induction of anti-oxidant defence systems, including reactive oxygen species (ROS)-scavenging enzymes such as catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and non enzamatic antioxidant [20-27].

Evidence has been presented that a rice annexin, interacted with various kinases, including receptor-like kinase, sterile-20 (Ste20)-like kinase and casein kinase using a tandem affinity purification approach and then identified the protein complexes [28]. Besides the MAPK cascade, Ca^{2+} signaling is another critical pathway triggered by environmental stimuli and developmental cues. Calcium-dependent protein kinases (CDPKs) are sernine/threonine protein kinases that are one of the best characterized Ca^{2+} sensors in plant. Previous studies suggested that CDPKs are involved in the responses of plants to various abiotic stresses, including salt, drought, hormonal stimuli,and oxidative stress [29-31]. However, the regulatory network regulate antioxidant defence in ABA signaling remain to be determined.

In this study, we investigated a putative annexin family gene in rice, designated *OsANN4*. Yeast two-hybrid assay showed that OsANN4 interacted with OsCDPK24. We also generated a series of transgenic rice plants, including the knocking down *OsANN4* through RNA interference (named RNAi plants), *OsANN4*pro::GUS and *OsANN4*-GFP lines. We found that the RNAi lines were more sensitive to ABA, and OsANN4 mediated oxidative stress response through ABA signaling pathways.

1　MATERIALS AND METHODS

1.1　Vector construction for rice transformation

Total RNA was extracted from 7-day-old seedlings of WT. The full-length *OsANN4* cDNA without the stop codon was amplified with primers P1 and P2 via reverse transcription (RT)-PCR. For sequencing and sub-cloning, the products were ligated into a T-vector first. After sequencing, the fragment was cut with *Xba*I and *Kpn*I and inserted in to the pCAMBIA2300OCS expression vector driven by a CaMV35S promoter. To construct the RNA interference vector, the 333-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. To construct the *OsANN4*pro::GUS vectors, total DNA was extracted from 7-day-old seedlings of WT, the 1794-bp DNA sequence upstream of the *OsANN4* start codon was amplified with primers P5 and P6. The sequence-confirmed fragment was inserted into the pCAMBIA1300-GUS between the *Pst*I and *Xba*I sites.

To examine the subcellular localization of OsANN4, the CaMV35S::OsANN4–GFP and CaMV35S::GFP vectors were constructed. The OsANN4 coding region was fused to GFP using the PMDC83 backbone between *Xba*I and *Kpn*I site to construct CaMV35S::OsANN4–GFP, and its expression was driven by the 35S promoter.

The plant transformation constructs were introduced into *Agrobacterium* EHA105 and then transformed into rice calli. Transgenic rice plants were generated as previously described [32,33].

1.2 Plant materials and stress treatment

The rice (Oryza sativa subsp. japonica) cultivar Nipponbare was used in this study，and as the wild type (WT) control in all experiment. Rice seeds were surface-sterilized with commercial bleach for 20 min followed by rinsing several times with sterile distilled water and grown on 1/2 MS medium. To measure the transcript level of *OsANN4*, the rice plants were grown in 1/2 MS culture solution in a greenhouse with a light/dark cycle of 16/8 h and 60-70% relative humidity at 28/25 ℃ (day/night). Tissue-specific expression of the annexin genes was analyzed at seedling stage after seeds germinated at 0 h, 2 h, 4 h, 8 h, 12 h, 24 h and shoot of seedlings 2- , 3- , 4-, 7-day-old and leaves at heading stage.

For stress testing, WT and RNAi transgenic rice seeds were using for the experiments. For ABA treatment assay, the seeds were planted on 1/2 MS medium, the 6-day-old seedlings were subjected to both 10 uM ABA or 100 mM H_2O_2.

1.3 β-glucuronidase (GUS) staining

To evaluate the expression patterns of *OsANN4*, we generated *OsANN4*pro::GUS transgenic rice plants. GUS staining was observed via histochemical β-glucuronidase staining. Three lines of positive transgenic rice plant samples were incubated in 5-bromo-4-chloro-3-indolyl-ß-glucuronic acid buffer in the dark at 37 ℃. After staining, the plant tissues were rinsed several times with 70% ethanol to remove surface dyes and chlorophyll.

1.4 RNA extraction, RT-PCR, and quantitative RT-PCR analysis

Total RNA from different tissues of rice plants was extracted using TaKaRa RNAiso Plus. Purified RNA (2 μg) was incubated with DNase (RQ1 RNase-Free DNase; Promega) at 37 ℃ for 15 min. First-strand cDNA was synthesised using PrimeScript™ First Strand cDNA Synthesis Kit (TaKaRa). The reaction products subjected to qRT-PCR using gene-specific primers P9 and P10. RT-PCR was performed using Taq DNA polymerase (Takara), and a 7500 Real-Time PCR instrument (Applied Biosystems) was used for qRT-PCR. *OsACTIN1* was used as an internal control for normalization of all data in this experiment. Three independent biological replicates were assayed.

1.5 Sub-cellular localization of OsANN4-GFP

To examine the subcellular distribution of OsANN4, 35S::OsANN4–GFP and 35S::GFP

vectors were constructed. The OsANN4 coding region was fused to GFP using the PMDC83 backbone to construct CaMV35S::OsANN4–GFP, and its expression was driven by the 35S promoter. The constructs were used for rice transformation, so the constructs were introduced into *Agrobacterium* EHA105 and then transformed into rice callus .Transgenic rice plants expressing OsANN4-GFP were generated as previously described[32,33]. The OsANN4-GFP protein was examined using confocal laser-scanning microscopy (Zeiss LSM510).

1.6 In situ localization of $O_2^{\cdot-}$ or H_2O_2 assay

To evaluate the localization of $O_2^{\cdot-}$ in situ, rice leaf discs (approximately 5 mm) were immersed in 6 mmol/L NBT (nitroblue tetrazolium) at 25 ℃ for 8 h in the light[29] (Schutzendubel et al., 2001) and then placedinto a boiling solution (60% ethanol, 20% ethylic acid, 20% glycerol) for 10 min. This process decolorized the leaves and revealed the dark blue insoluble formazan produced by the reaction of NBT with $O_2^{\cdot-}$. The samples were imaged after cooling.

DAB staining was performed following a published method with some modifications. Seven-day-old rice seedlings were treated with a 50 ℃ heat shock for 30min, and then 3−5 mm leaf sections were detached and immersed in a solution containing $1 mgml^{-1}$ 3,3′-diaminobenzidine (DAB) prepared in HCl-acidified (pH 3.8) water at 25 ℃ for 14 h. The samples were then incubated in a boiling solution (60% ethanol, 20% glycerol and 20% ethylic acid) for 10 min. The samples were observed and imaged under a light microscope.

1.7 Yeast two-hybrid analysis

The *OsANN4* coding sequences were cloned into the BD vector with the *Nde*I and *Eco*RI sites. The OsCDPK24 coding sequence was cleaved into several fragments for further subcloning into AD vectors. The expression vectors pGADT7–OsCDPK24 and pGBKT7–OsANN4 were co-transformed into the yeast strain AH109 (Clontech) with the lithium acetate method (Clontech Yeast Protocols and book). Colonies were then transferred to selective medium lacking leucine, tryptophan, histidine and adenine (SM-LWHA), and this result was used for the first screening. The selective medium lacking leucine and tryptophan (SM-LW) was used for the second screening. A positive control interaction between the 53 protein and the SV40 protein and a negative control interaction between the Lam protein and the SV40 protein were observed.

2   RESULTS

2.1 Tissue-specific expression of OsANN4

Previous studies have demonstrated that the annexin family genes are expressed differentially in various tissues during different developmental stages [8,11]. In this study, we evaluated the expression patterns of OsANN4 in various tissues with the GUS reporter gene. We obtained 16 independent positive transgenic rice lines expressing *OsANN4*pro::GUS, and three lines were used to in the following experiments. We perform GUS staining analysis then and the results showed

that the strongest signals were found in the coleoptide, weak signals were found in the tip of the lemma .GUS staining was found in coleoptide and embryo in the 2-day-old seedlings, but in the 3-and 4-day-old seedlings GUS staining was only found in coleoptide. However, there was no GUS staining found in roots, stems, or in floral parts. (Fig.1A)

Based on the results, we further analyzed the expression of *OsANN4* with quantitative real-time PCR during the seedling growth after germinated at 0 h, 2 h, 4 h, 8 h, 12 h, 24 h and seedlings of 2-, 3-, 4-, 7-day-old and leaves during heading stage. Quantitative real-time PCR showed that the transcript abundance of OsANN4 increased as the seedlings growth and it was also showed increase in transcript level from 3d to 4d seedlings (Fig .1B). According to the semi-quantitative RT-PCR, the transcript level increased from 0d to 7d, and the transcript level is significantly high at 7d and during the heading stage (Fig.1C). The differences of expression pattern in individual organs and stages suggested the OsANN4 may have potential spatial functional specificity and function whole life of rice.
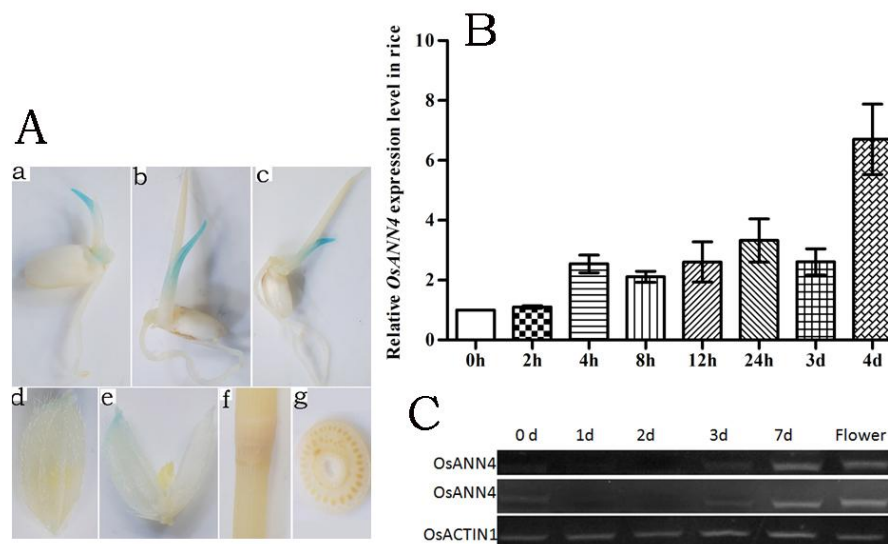

Fig. 1 OsANN4 expression patterns in rice.

2.2 Knocking down *OsANN4* resulted in transgenic plants sensitivity to ABA

To examine the effect of knocking down *OsANN4* on ABA response, 20 independent transgenic RNAi lines were generated. Semi-quantitative RT-PCR showed that *OsANN4* was less expression in all RNAi lines. WT and three independent RNAi lines seeds were planted on 1/2 MS medium with or without 10 uM ABA. There was no more difference between the WT and RNAi plants. However, when they were planted on 1/2 MS medium containing 10 uM ABA, though there was no significant difference in germination rates between WT and the RNAi lines, the RNAi seedlings showed obvious slower growth compared to WT at the first six days (Fig.2). This result indicates that the RNAi seeds were hypersensitive to ABA at the early seedling stages.

Fig.2 OsANN4 knockdown resulted in increased sensitivity to ABA.

2.3 *OsANN4* is involved in ABA induced stress response.

More and more evidences suggested that ABA enhanced stress tolerance were associated with the induction of antioxidant defense system so as to protect plant cell against oxidative damage. In ABA signaling, ROS are important signal molecules. To assess the effect of knocking down OsANN4 on response, we used the 5-day-old seedlings of WT and RNAi lines to assay the activities of antioxidant enzymes and the production of H_2O_2. The RNAi lines showed slightly decreased activities of CAT compared to WT. Interestingly, when treated by 10 uM ABA for 5 hours, the WT showed decreased activities of CAT, while the RNAi lines showed increased activities of CAT contrast to no ABA present (Fig.3A). CAT is an important enzyme could catalyses H_2O_2 to H_2O and O_2, and protects plant cells from oxidative damage by ROS. To examine whether ROS are involved in *OsANN4* modulate ABA signaling, the levels of H_2O_2 in WT and RNAi lines were measured. To assay H_2O_2 content, 5-day-old rice seedlings treated by 10 uM ABA for 5 hours and then subjected to DAB staining. Without ABA treatment, there was no visible difference in mesophyll cells of leaves between the WT and the RNAi lines. However, there were more darkly stained patches in mesophyll cells of leaves of RNAi lines after ABA treatment (Fig.3B). The result indicated that there was an increase level of H_2O_2 or O_2^- in RNAi lines. Furthermore, we used both 10 uM ABA and 100 mM H_2O_2 to treat 5-day-old seedlings, both the WT and the RNAi lines showed increased activities of CAT (Fig.3A). The results indicated that ABA induced the overproduction of H_2O_2 and a certain content of H_2O_2 would start OsANN4 to modulate CAT activity to scavenge ROS as a stress defense factor. Though ABA induced stress response when OsANN4 was knockdown, the activity antioxidant defense enzyme was not high enough to confer the stress. The result indicated that *OsANN4* was involved in ABA induced stress response.
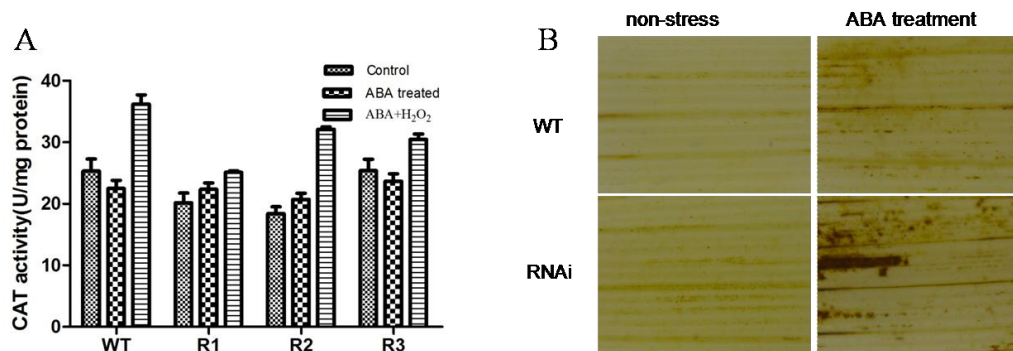
Fig.3 CAT acticity and DAB staning assay.

2.4 OsANN4 may interact with OsCDPK24

200　　　We assayed the interactions between rice annexins and kinases using yeast two-hybrid. The OsANN4 coding sequences were cloned into the BD vector between the *Nde*I and *Eco*RI sites. The OsCDPK24 coding sequence was cut into several fragments for further sub-cloning into AD vectors. The expression vectors pGADT7–OsCDPK24 and pGBKT7–OsANN4 were co-transformed into the yeast strain AH109. Yeast transformants grow normally on SM-LW. The

205　　normal growth of yeast on SM-LWHA and its ß-galactosidase activity were used as indicators of interaction. His autotrophy was restored on SM-LWHA when OsANN4 was co-transformed with OsCDPK24 (Fig.4). However, the cells was not able to grow on SM-LWHA when OsANN4 was co-transformed with pGADT7 or pGBKT7. This result indicated that OsANN4 might specifically interact with OsCDPK24.
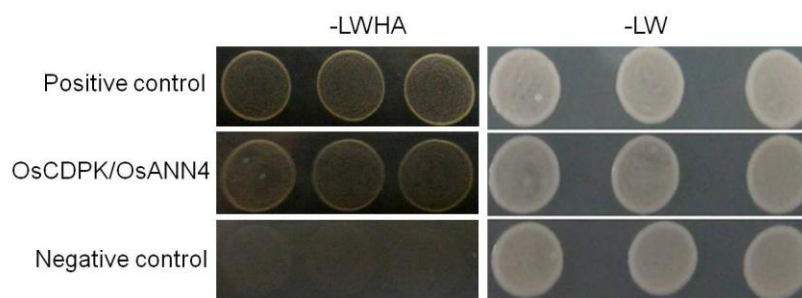

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Fig.4 Yeast two-hybrid system detecting the OsANN4 and OsCDPK24 interaction

2.5 OsANN4 subcellular distribution

　　　To determine the subcellular localization of OsANN4, 35Spro::OsANN4-GFP and 35Spro::GFP were introduced individually into *Agrobacterium tumefaciens* EHA105. The

215　　*Agrobacterium* was then used to transform rice callus. Twenty-five independent 35Spro::OsANN4-GFP lines were obtained, and 3 lines of each were used for further analyses. The fluorescence of the GFP control was observed throughout the rice root tip cells. Conversely, the fluorescence signals of the OsANN4-GFP fusion proteins driven by the 35S promoter were observed at the cell periphery but not in the nucleus in the elongation zone of rice root tip cells
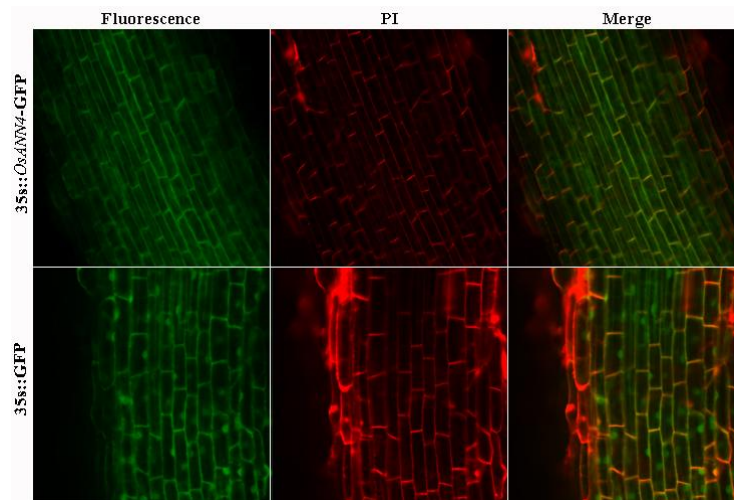
220　　(Fig.5).

Fig.6 Subcellular distribution of OsANN4-GFP in rice roots

3　Discussion

Annexins are traditionally received Ca^{2+}-dependent phospholipids-binding proteins, which usually contain a characteristic type II Ca^{2+}-binding residue in each corresponding repeat in vertebrates. However, in plant annexins, the type II Ca^{2+} binding residues are absent in repeats 2 and 3, what's more, there is no type II Ca^{2+} binding residues found in OsANN4. However, our results indicate that OsANN4 is capable of binding in a Ca^{2+}-dependent manner to negatively charged phospholipids. Our results could be taken as evidence that annexins can also function in their Ca^{2+}-free conformation in a hitherto unknown intricate fashion, thus increasing the functional diversity of annexins.

A previous study suggested that a rice annexin could interacts with various kinases, including receptor-like kinase, sterile-20 (Ste20)-like kinase, and casein kinase. The Ste20 kinase is a MAKKK related to mating and osmotic stress responses in yeast. Since the CDPK and MAPK path ways are often response to the same environmental stimuli, CDPK(calcium-dependent protein kinase) may interact with annexins. As we predicted, OsANN4 could interact with OsCDPK24. Furthermore, the result also offered a support that there might exist a potential crosstalk between the two significant protein kinase families and annexins. Recently, the complex mechanisms regulatory network attracts extensive attention. In rice, a recent study showed that OsCPK12 can induce the expression of the antioxidant genes OsAPX2 and OsAPX8 under salt stress, and reduce the salt-induced accumulation of H_2O_2 [31]. These results suggested that OsCPK12 positively regulated ROS detoxification by controlling the expression of antioxidant genes. However, whether CDPKs are involved in ABA-induced antioxidant defense remains to be determined. ZmCPK11 had been proved to be involved in abscisic acid-induced antioxidant defense and functions upstream of ZmMPK5 in abscisic acid signaling in maize [20]. A rice C2H2-type zinc finger protein, ZFP36, was reported to be a key player involved in abscisic acid-induced antioxidant defense and oxidative stress tolerance in rice [35]. However, the regulatory network regulating antioxidant defense in ABA signaling remain to be determined.

Many ABA-induceible genes contain a conserved ABA-responsive element (ABRE). Sequence analysis of *OsANN4* shows that there is an ABRE at -241 promoter region of *OsANN4*. *OsANN4* knockdown results in decreased CAT activities, but the activities can be recovered when exogenous ABA was added. Though ABA induces stress response when *OsANN4* was knockdown, the activity antioxidant defense enzyme did not high enough to confer the stress. The result indicate that OsANN4 involved in ABA induce stress response. Ca^{2+} and CDPK are important signaling intermediate components in ABA-induced antioxidant defense. OsANN4 is Ca^{2+}-dependent phospholipids-binding proteins that interact with OsCDPK24.

4 Conclusion

In this paper, we investigated a putative annexin family gene in rice, designated *OsANN4*. We showed that OsANN4 could bind phospholipids in a Ca^{2+}-dependent manner and examined the ATPase of OsANN4 in vitro. We also generated a series of transgenic rice plants, including *OsANN4*pro::GUS and OsANN4-GFP lines. We found that the RNAi lines were sensitive to ABA, and *OsANN4* mediated oxidative stress response through ABA signaling pathways.

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OsANN4 耐受 ABA 信号诱发的胁迫并调控抗氧化物的积累

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摘要：膜联蛋白是一类进化保守的多基因家族，能够以 Ca2+依赖的方式与磷脂结合，广泛地分布于动物和植物中。OsANN4 是水稻膜联蛋白家族的一员，目前，OsANN4 在胁迫响应以及信号通路中的作用机制还不清楚。GUS 活性分析以及实时荧光定量 PCR 结果显示 OsANN4 的表达具有组织特异性，在幼苗生长阶段高表达。OsANN4 干涉转基因水稻在种子萌发期及幼苗生长早期呈现出对 ABA 敏感的表型，而且在 ABA 存在的情况下，其对 H2O2 更为敏感。这表明，OsANN4 响应 ABA 信号，并调控过氧化物的积累。此外，酵母双杂交结果显示 OsANN4 与 OsCDPK24 可能存在相互作用。这为膜联蛋白响应非生物胁迫提供了一个可能的调控网络。

关键词：分子生物学；膜联蛋白；钙结合活性；ABA 信号；水稻

中图分类号：Q751