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ORIGINAL ARTICLE

Overexpression of OsCOIN, a putative cold inducible zinc finger protein, increased tolerance to chilling, salt and drought, and enhanced proline level in rice

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Abstract Rice (*Oryza sativa* L.) plant is sensitive to chilling, particularly at early stages of seedling development. Here a novel cold-inducible gene, designated *OsCOIN* (*Oryza sativa* cold-inducible), was isolated and characterized. Results showed that OsCOIN protein, a RING finger protein, was localized in both nuclear and cytoplasm membrane. *OsCOIN* is expressed in all rice organs and strongly induced by low temperature, ABA, salt and drought. Overexpression of *OsCOIN* in transgenic rice lines significantly enhanced their tolerance to cold, salt and drought, accompanied by an up-regulation of *OsP5CS* expression and an increase of cellular proline level.

Keywords Cold inducible gene · Abiotic stress · Transgenic rice · RING finger protein · OsCOIN

Introduction

Rice, an important stable food crop with its relative small genome, has been used as a monocotyledonous model plant for dissecting genetic networks of biotic and abiotic responses in cereal crops. Cold (ranges 0–12°C) stress is one of the many obstacles for production of rice (*Oryza sativa* L.) cultivation in temperate zones and high-elevate environments. An important breeding objective in these regions is to develop cultivars tolerant to low temperatures at critical growth stages (Nakagahra et al. 1997). Rice plants are injured at the seedling stage when they are grown in early spring in temperate or subtropical environments. The types of low-temperature effects on seedlings can be manifested as poor germination, slow growth, discoloration or yellowing, withering after transplanting, reduced tilling, and stunted growth (Andaya and Mackill 2003).

ABA as a phytohormone plays a critical role in response to various stress signals. The application of ABA to plant mimics the effect of a stress condition. Numerous studies have shown that ABA is essential for the normal adaptive response to water stress imposed by drought or high salinity (Koorneef et al. 1984; Leon-Kloosterziel et al. 1996; Xiong et al. 2002). Many of the biochemical and physiological changes under stress result from ABA-induced changes in gene expression patterns (Ramanjulu and Bartels 2002; Shinozaki and Yamaguchi-Shinozaki 2000). The importance of ABA in low-temperature adaptation is somewhat controversial (Thomashow 1999). The endogenous ABA level increases under low-temperature conditions and application of exogenous ABA enhances cold tolerance of non-acclimatized plants (Lang et al. 1994; Mantyla et al. 1995). Gene expression induced by ABA often relies on the presence of *cis* acting element called ABRE (Finkelstein et al. 2002; Shinozaki and Yamaguchi-Shinozaki 2000; Thomashow 1999; Uno et al. 2000). All three common abiotic stresses, drought, low temperature and high salinity, cause an accumulation of compatible solutes and antioxidants, such as sugars, proline (Hasegawa et al. 2000; Fukutaku and Yamada 1984).

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There are some overlaps in the regulation pathways of gene expression between different environmental stresses (Rabbani et al. 2003).

Zinc finger proteins as members of transcriptional factors were further grouped into the subfamilies of TFIIIA, WRKY, Dof, LIM, and RING finger. In plants zinc finger proteins are involved in growth, development and responses to environmental stresses. bZIP proteins contain a region of basic amino acids followed by a region containing at least three to four repeats of Leu or another hydrophobic amino acid. The hydrophobic region mediates homodimer formation, whereas the basic region is involved in DNA binding. A notable exception is the cold-induced LIP19 in rice, which does not bind to DNA directly but rather binds to OsOBF1, another new bZIP protein, to form a heterodimer (Aguan et al. 1993; Rabbani et al. 2003; Shimizu et al. 2005). ZFP245, a C2H2-type zinc finger protein, probably plays a role in cold and drought responses in monocots (Huang et al. 2005; Kim et al. 2001).

The promoters of stress responsive genes have typical *cis*-regulatory elements like DRE/CRT, ABRE, MYCRS/MYBRS and are regulated by various upstream transcriptional factors. These upstream transcription factors fall in the category of early genes and are induced within minutes of stress. Our recent studies suggest that MYB3R-2 is involved in the gene control network in plant (Dai et al. 2007). The transcriptional activation of some of these genes including *RD29A* has been well worked out. The transcription factors CBF1, 2 and 3, are cold responsive and in turn bind DRE/CRT elements and activate the transcription of various stress responsive genes (Mahajan and Tuteja 2005). The expression of the stress responsive genes leads to physiological responses (Finkelstein et al. 2002).

In the present study, we have cloned and characterized *OsCOIN* (*Oryza sativa* cold-inducible), a novel cold-inducible gene in rice. In addition, we demonstrated that the expression of *OsCOIN* can be induced by cold (4°C), exogenous ABA, salt, and drought treatments. Finally, we showed that overexpression of *OsCOIN* in transgenic rice lines significantly enhanced the proline content of the cells and improved tolerance to cold, salt and drought treatment.

Materials and methods

Plant materials and growth conditions

All rice materials used in the experiments were *Oryza sativa* L. cv Zhonghua 10. All rice plants (both wild type and transgenic) were grown in fields in natural conditions or in the greenhouse at 28°C/25°C (day/night) with a 16 h photoperiod under a relative humidity of 50%. For physiological experiments, the seeds of T_2 transgenic and wild type rice were allowed to germinate in 30°C in darkness for 2 days, and then transferred to agar plates containing half of MS (Murashige and Skoog) medium and continued to culture for 12 days at 25–28°C with a 16 h photoperiod. To measure the *OsCOIN* expression in different rice organs 2-week-old seedlings, young roots from trifoliate stage and other organs from adult rice were collected and used in RT-PCR.

Isolation of *OsCOIN* and construction of vectors

Total RNA from 2-week-old rice seedlings was isolated using Trizol Reagent (Invitrogen life technologies, Carlsbad CA 92008, USA), and then reverse transcribed as described (RT-PCR kit, Promega corporation, Madison, WI, USA). The first strand cDNAs were used as templates for PCR amplification with 5′-GGGGTACC ATG AGC TCT CTA TGC CCC TTT GCC A-3′ (*Kpn*I site underlined) and 5′-GGGGATCC CTT GTC ATC CAA TTG TTT TTG TAG A-3′ (*Bam*HI site underlined) as primers. PCR was performed with LA Taq DNA polymerase (Takara, Japan) as follows: preheating at 94°C for 5 min, then 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The PCR products were cloned into pGEM-T easy (Promega, Madison, WI, USA) and sequenced. The *OsCOIN* gene fragment was then obtained by digestion with *Kpn*I and *Bam*HI and cloned downstream of a maize ubiquitin promoter in the pUN1301 vector (Ge et al. 2004). PCR and enzyme digestion were used to identify the positive clones.

Subcellular localization

The *OsCOIN* cDNA was amplified with the primers 5′-GCTCTAGA ATG AGC TCT CTA TGC CCC TTT GCC A-3′ (F) (*Xba*I site underlined) and 5′-GGGGTACC CTT GTC ATC CAA TTG TTT TTG TAG A-3′(R) (*Kpn*I site underlined). The PCR products were digested with *Xba*I and *Kpn*I, and ligated with *Xba*I and *Kpn*I double-digested *pGFP221* to created *pGFP-OsCOIN*, in which the coding region of the *OsCOIN* gene was fused to the N terminus of the GFP in frame, under the control of the cauliflower mosaic (CaMV) 35S promoter. The fusion construct and the GFP control vector were transformed into the onion epidermis cells by particle bombardment according to the protocol described (Varagona et al. 1992). The transformed cells were cultured on MS medium for 24 h and observed under a confocal microscope (ZEISS, Germany).

Generation of *OsCOIN* transgenic rice lines

Rice calli were induced on scutella from germinated seeds and transformed with strain EHA 105 of *Agrobacterium*

tumefaciens containing the desired binary vector, as described (Xu et al. 2005). Transgenic rice plants were selected in half-strength MS medium containing 75 mg L^{-1} hygromycin (Sigma). Hygromycin-resistant plants from calli, designated as the T0 generation, were transplanted into soil and grown in a greenhouse at 28°C. To confirm the transgenic rice plant further, seeds of the T0 generation were germinated in half-strength MS medium containing 75 mg L^{-1} hygromycin and analyzed by GUS staining. T2 generation of transgenic rice plants was used in subsequent experiments.

Southern blot analysis

Genomic DNA (20 μg), purified from transgenic or wild type rice plants (T2 generation), was digested with *Eco*RI or *Hin*dIII at 37°C for 20 h and then separated by 0.8% agarose gel. The genomic DNA was transferred and cross-linked onto a nylon membrane (Hybrid N^+; Amersham) according to a protocol described (Sambrook et al. 1989). The *GUS* probe was synthesized by PCR with primers 5′-GCA GTG TAC GTC CTG TAG AAA CCC-3′ (F) and 5′-CAA AGC CAG TAA AGT AGA ACG GT-3′ (R) and labeled with [^{32}P] dCTP (China Isotope, Beijing). After pre-hybridization of about 6 h, the heat-denatured probe was added and then hybridized for another 20 h at 65°C. The membrane was washed twice with 2× SSC plus 0.1% SDS at 65°C, and once with 1× SSC plus 0.1% SDS at 65°C. The membrane was exposed to the X-ray film (Kodak, Rochester, NY) at −70°C for 1 day or more.

Four-degrees cold, ABA, NaCl and drought treatments

Rice seeds were sterilized and then germinated in half of MS agar medium before the treatments as described by Hur et al. (2004). To examine the time course of *OsCOIN* expression under cold stress, the cold treatment was accomplished by transferring the chamber with the 2-week-old seedlings of WT rice plants into a prechilled (4°C) Low Temperature Biochemical Incubator (BTI100, LEAD TECH, USA) (http://www.sciequip.com.cn/subproduct.asp?SubCategoryID=773) according to Pramanik and Imai (2005). At different time points samples were withdrawn from the Low Temperature Biochemical Incubator and frozen in liquid nitrogen immediately for subsequent RNA extraction. To examine the expression of *OsCOIN* under exogenous ABA, salt and drought stress, 2-week-old seedlings of the wild type rice (T2 generation) were treated in medium in the chamber with different concentrations of ABA, or NaCl, or PEG6000 for 6 h. Once the optimal concentrations for ABA, NaCl and PEG6000 have been determined, the time courses of *OsCOIN* induction by ABA, NaCl or PEG6000 have also been examined. To investigate the effect of overexpression of *OsCOIN* gene on salt tolerance, the seeds of both wild type and *OsCOIN* transgenic rice (T2 generation) were submerged into 1/2 MS medium with 250 mM NaCl and germinated for 20 days. To examine the effect of overexpression of *OsCOIN* gene on cold tolerance, 2-week-old rice seedlings of both wild type and *OsCOIN* transgenic rice (T2 generation) were exposed to chilling temperature (4°C) for 60, 72, and 84 h after the MS agar had been washed off, and then returned to the normal growth conditions to allow their recovery. To examine the effect of overexpression of *OsCOIN* gene on drought tolerance, 2-week-old seedlings of both wild type and *OsCOIN* transgenic rice (T2 generation) were submerged into the medium with 20% of PEG6000 for 24 h after the MS agar had been washed off.

RT-PCR analysis

Total RNA were extracted from the 2-week-old seedlings of the wild type or transgenic rice plants (T2 generation) describe above using Trizol Reagent (Invitrogen life technologies, Carlsbad CA 92008, USA), and then reverse transcribed as described (RT-PCR kit, Promega corporation, Madison, WI, USA). For the first-strand cDNA synthesis, 2 μg of total RNA was reversed-transcribed in a total volume of 20 μl reaction buffer that contained 10 ng of oligo (dT)-**18** primer, 2.5 mM dNTP, and five units of AMV reverse transcriptase (Promega, Madison, USA). PCR was performed in a 20 μl solution containing a 1 μl of the cDNA template, 0.1 μM of gene-special primers, 2 mM dNTPs, one unit of LA Taq DNA polymerase. The reaction included an initial 5 min denaturation at 94°C, followed by 20–40 cycles of PCR (94°C 1 min, 56°C 30 s, 72°C 1 min), and a final 10 min extension at 72°C. The PCR products were separated on a 0.8% agarose gel. The primers used for gene-specific PCR were listed as follows: (a) *OsNAC6*: 5′-CAT GGC CGG TGA ACT TTG AC-3′ (F), 5′-CTC GTC GTC GTT CAG TCC AG-3′ (R); (b) *OsLti6*a: 5′-AAT ACT GCG AGA GAA ATT AAT CA-3′ (F), 5′-TAA GAG GGG AGC TTA TTC ACA C-3′(R); (c) *OsLti6b*: 5′-GCC TTA AAT TGG AGC TCA GTC-3′ (F), 5′-GTG CAG AAG ATA AAC TGG AGA A-3′ (R); (d) *OsP5CS*: 5′-AAG ATG GAA GAT TGG CTT TGG GCA G-3′ (F), 5′-TCT CGT GTA GGT AGA GGA GGC ATG A-3′ (R); (e) *OsTubulin*: 5′-TCA GAT GCC CAG TGA CAG GA-3′ (F), 5′-TTG GTG ATC TCG GCA ACA GA-3′ (R); (f) *OsCOIN*: 5′-ATG AGC TCT CTA TGC CCC TTT GCC A-3′ (F), 5′-CTT GTC ATC CAA TTG TTT TTG TAG A-3′ (R).

Determination of cellular proline levels

Two-week-old seedlings (0.5 g) from wild type and *OsCOIN* transgenic rice (T2 generation) with or without

cold treatment were homogenized in 2 ml of 3% aqueous sulfosalicylic acid and centrifuged. Free proline amount was measured by using spectrophotometer as described by Bates et al. (1973) and reported as μmol g FW^{-1}.

Results

Isolation and characterization of *OsCOIN* gene

To isolate the cold-inducible gene (GenBank AK104280) in rice (*Oryzia sativa* L.), the full-length *OsCOIN* cDNA was amplified by RT-PCR, cloned into pGEM-T easy and sequenced. The cDNA of *OsCOIN* consists of 1,593 nucleotides, which include a 1,089-bp ORF (open reading frame) from 143 to 1,231 bp. The ORF encodes a putative protein of 363 amino acids with a predicted molecular mass of 42 kDa and a pI of 5.25. Analysis of the deduced amino acid sequence reveals that this protein may contain a conserved cysteine-rich domain of 40–60 residues (called "RING finger") (Fig. 1a) that binds two atoms of zinc. The 3D structure of the zinc ligation system is referred to as "cross-brace" motif. This atypical conformation is also shared by the FYVE (PDOC50178) and PHD (PDOC50016) domains. Yeast hybrid assay determined that OsCOIN protein had no trans-activation activity (data not shown). Bioinformatic analysis revealed that the promoter of *OsCOIN* gene had the cold, ABA, salt and drought responsive elements (Fig. 1b). Multiple-alignment of OsCOIN with other related proteins showed that the deduced amino acid sequence of OsCOIN has more than 61% identity to gi21207099 (*Zea mays*), gi30694045 (*Arabidopsis thaliana*), gi47104584 (*Lycopersicon esculentum*) in the public database (Fig. 1c), indicating that these four plant proteins may belong to the same family.

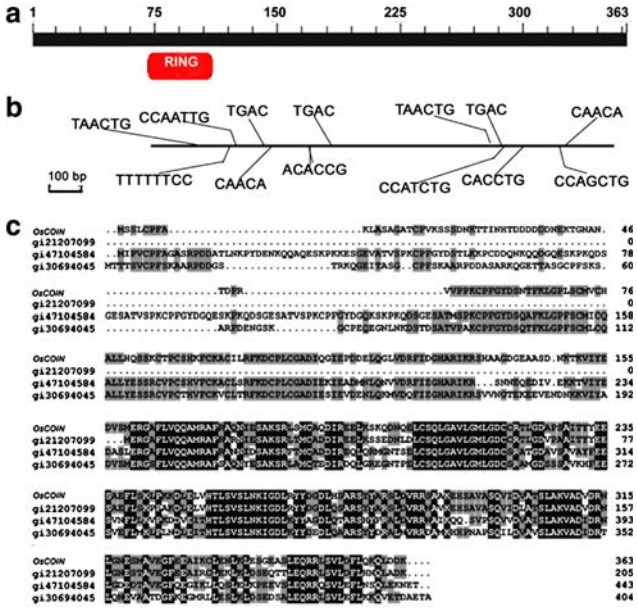

Fig. 1 Characterization of *OsCOIN* gene (GenBank AK104280). **a** Putative conserved domains were analyzed with the protein–protein BLAST tool of NCBI (http://www.ncbi.nlm.nih.gov). **b** Analysis of the *OsCOIN* promoter sequence using http://www.dna.affrc.go.jp/sig-scan/signal. TAACTG: MYB recognition sequence; CCANNTG (*N* = A/T/G/C): MYC recognition sequence; TGAC: W-Box; TTTTTTCC: *cis*- and *trans*-acting elements; CAACA/CACCTG: AP2-like and B3-like motif. **c** Alignment of the deduced amino acid sequences of OsCOIN (AK104280), AK071071, gi21207099, gi30694045, gi47104584 using DANMAN

OsCOIN protein is a nuclear and cytoplasma protein

The subcellular localization of OsCOIN had been studied in vivo using the transient transfection assay. Figure 2d showed that the OsCOIN–GFP fusion protein was located at the nuclear and the cytoplasm membrane under a confocal microscope. Under the same conditions, comparatively, GFP alone was distributed in the whole cell (Fig. 2b). The results suggested that OsCOIN was a protein located at nuclear and cytoplasm membrane.

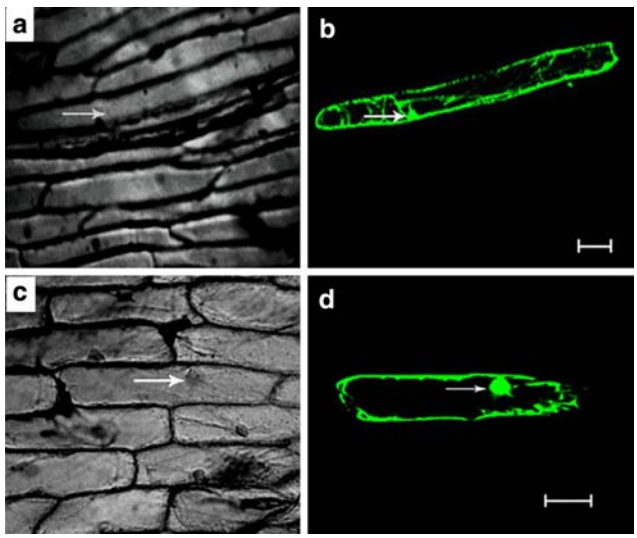

Fig. 2 Subcellular localization of the OsCOIN protein in onion epidermal cells. Green fluorescent protein (**b**) and OsCOIN::GFP protein (**d**) in onion epidermal cells (*arrow* indicated nuclear). GFP or OsCOIN::GFP fusion was expressed transiently under the control of the CaMV35S promoter in onion epidermal cells. Bars: 50 μM

OsCOIN expression in various rice organs and induction by cold, ABA, salt and drought

The expression of the *OsCOIN* gene in various organs of the wild-type rice plants were analyzed by semi-quantitative RT-PCR using *tubulin* as an internal control. As shown in Fig. 3a, the *OsCOIN* gene is expressed in all organs of the wild-type rice plants indicating that this gene could be a house-keeping gene.

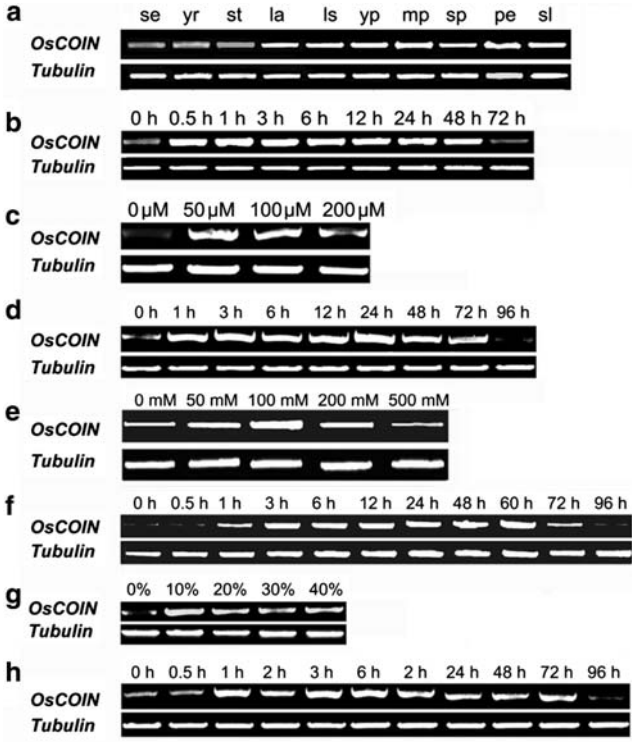

Fig. 3 *OsCOIN* expression in various rice organs and induction of *OsCOIN* by various stress conditions. **a** Total RNAs from different organs of wild-type rice plants (*Oryzia sativa* L.) were purified and reverse-transcribed. The resultant cDNAs were used as templates for RT-PCR using *Tubulin* as a template control. *se* seedling; *yr* young root; *st* stem; *la* lamina; *ls* leaf sheath; *yp* young panicle; *mp* mature panicle; *sp* stem primodia; *pe* pedestal; *sl* stipital leaf. **b** The time course of the *OsCOIN* induction by 4°C cold treatment. **c** The induction of *OsCOIN* expression after 6-h treatment with different concentrations of exogenous ABA. **d** The time course of the *OsCOIN* induction by 50 μM of ABA. **e** The induction of *OsCOIN* expression after 6-h treatment with different concentrations of NaCl. **f** The time course of the *OsCOIN* induction by 100 mM of NaCl. **g** The induction of *OsCOIN* expression after 6-h treatment with different percentages of PEG6000. **h** The time course of the *OsCOIN* induction by 10% of PEG6000. Total RNAs were purified from the 2-week-old seedlings of the wild type rice (T2 generation) after the above treatments and reverse-transcribed. The resultant cDNAs were used as templates for RT-PCR using *Tubulin* as a template control. PCR was performed at an annealing temperature of 56°C and 38 cycles were used for *OsCOIN* and 28 cycles were used for *tubulin*. Experiments were repeated at least three times with similar results. In all the experiments no PCR band was observed in a water control

We then examined the expression of *OsCOIN* under cold stress in wild type rice. As shown in Fig. 3b, a huge increase in *OsCOIN* mRNA was already observed in rice seedlings (WT) after being exposed to cold (4°C) for only 30 min and this high level of expression was maintained for at least 48 h. At 72 h the *OsCOIN* expression declined to the level before the cold stress.

To examine the effect of exogenous ABA on *OsCOIN* expression, 2-week-old seedlings of wild type rice were immersed into the solutions with different concentrations of exogenous ABA. As shown in Fig. 3c, 50 μM of ABA induced the maximum *OsCOIN* expression. Analysis of the time course of *OsCOIN* induction by ABA showed that exogenous ABA (50 μM) resulted in a rapid induction of *OsCOIN* mRNA in the 2-week-old rice seedlings, reaching the peak around 1 h and maintaining the maximum level for up to 72 h, and then returned to the normal level after 96 h (Fig. 3d).

Figure 3e showed that 100 mM of NaCl induced the maximum *OsCOIN* expression in the 2-week-old rice seedlings. Analysis of the time course of *OsCOIN* induction by NaCl showed that 100 mM of NaCl treatment resulted in a rapid induction of *OsCOIN* mRNA in the 2-week-old rice seedlings, reaching the peak around 3 h and maintaining the maximum level for up to 60 h, and then returned to the normal level after 96 h (Fig. 3f).

To test the effect of the drought treatment on *OsCOIN* expression, 2-week-old seedlings of wild type rice were immersed into the solutions with different concentrations of PEG6000. Figure 3g showed that the maximum *OsCOIN* expression was induced by 10% of PEG6000. Analysis of the time course of *OsCOIN* induction by PEG6000 showed that 10% of PEG6000 treatment resulted in a very rapid induction of *OsCOIN* mRNA in the 2-week-old rice seedlings, reaching the peak around 1 h and maintaining the maximum level for up to 72 h, and then returned to the normal level after 96 h (Fig. 3h).

Identification of *OsCOIN* transgenic rice plants

To explore its function, the *OsCOIN* gene driven by an ubiquitin promoter together with a *GUS* gene as a marker was transformed into rice plants by the *Agrobacterium* transformation approach as described in experimental procedures. Southern blot with a probe of *GUS* gene showed that one hybridized band at different position (3, 4, and 6 kb for *Eco*RI digestion, and 2, 2.5, and 6 kb for *Hin*dIII) was presented in each of the three independent transgenic rice lines (Fig. 4a), indicating that the exogenous *OsCOIN* gene was integrated into the rice genome. The expression level of *OsCOIN* in T2 transgenic plants was analyzed by RT-PCR. As shown in Fig. 4b, *OsCOIN* expression in the three *OsCOIN* transgenic rice lines was stronger than that in the wild type rice.

OsCOIN transgenic rice is more tolerant to cold, salt and drought

To examine the effect of overexpression of *OsCOIN* gene on cold tolerance, 2-week-old rice seedlings of both wild type and *OsCOIN* transgenic rice (T2 generation) were exposed to chilling temperature (4°C) for 60, 72, and 84 h after the MS agar had been washed off, and then returned to the normal growth conditions to allow their recovery. After 2 weeks in

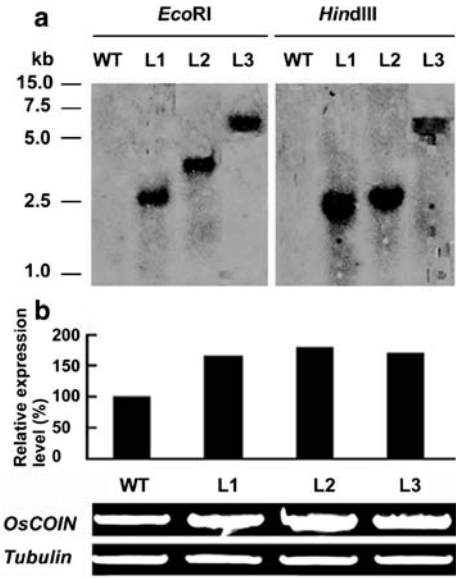

Fig. 4 Characterization of the *OsCOIN* transgenic rice. **a** Southern blot analysis. The genomic DNA from the three independent transgenic rice lines (*L1*, *L2* and *L3*) were digested with *Eco*RI or *Hin*dIII, separated by gel electrophoresis and blotted onto nylon membranes. Blots were probed with α-^{32}P-dCTP-labeled *GUS* gene. The expected sizes of the bands are 3, 4, and 6 kb for *Eco*RI digestion, and 2, 2.5, and 6 kb for *Hin*dIII digestion. **b** The levels of *OsCOIN* expression in wild type and *OsCOIN* transgenic rice lines. Total RNAs from WT and *OsCOIN* transgenic rice were analyzed by RT-PCR. *Tubulin* was used as a template control. PCR was performed at an annealing temperature of 56°C and 38 cycles were used for *OsCOIN* and 28 cycles were used for *tubulin*. Experiments were repeated at least three times with similar results. No PCR band was observed in a water control

the greenhouse, the *OsCOIN* transgenic plants treated for 60, 72, and 84 h, re-grew 76.2, 71.4 and 50% respectively, while the wild type rice under the same conditions re-grew only 52.4, 22.2 and 14.8%, respectively (Fig. 5a, b).

To analyze the effect of overexpression of *OsCOIN* gene on tolerance to drought, the 2-week-old seedlings of wild type and the *OsCOIN* transgenic rice plants were submerged in the solutions containing 20% of PEG6000 for 24 h after the MS agar had been washed off. As shown in Fig. 6a, b, 92% of the leaves from the WT rice were rolled while only 8% of the leaves from the transgenic rice (T2 generation) were rolled. After 2 weeks culturing in the greenhouse for their recovery, the *OsCOIN* transgenic plants treated for 12 and 24 h, re-grew 85 and 60%, respectively, while the wild type rice under the same conditions re-grew only 40 and 7.5%, respectively (Fig. 5c, d).

To examine the effect of overexpression of *OsCOIN* gene on salt tolerance, the seeds of both wild type and *OsCOIN* transgenic rice (T2 generation) were submerged in 1/2 MS medium with 250 mM NaCl and germinated for 20 days. As shown in Fig. 6c, d, the seeds from the *OsCOIN* transgenic rice germinated faster (35%) and grew taller than that from the wild type rice.

Expression of cold-responsive genes in *OsCOIN* transgenic rice

To investigate the possible gene regulation mechanisms of cold tolerance mediated by *OsCOIN*, we analyzed the expression of several known cold-induced genes by RT-PCR. As demonstrated in Fig. 7a, the expression levels of *OsNAC6*, *OsP5CS* and *OsLti6b* were increased in *OsCOIN* transgenic rice compared with the levels in wild type rice after cold treatment (4°C) for 24 h. Interestingly, the expression of *OsLti6a* in transgenic rice remains the same as in wild type rice.

Determination of cellular proline levels

The levels of proline were measured as shown in Fig. 7b. Proline contents in the 2-week-old seedlings of the wild type and *OsCOIN* transgenic rice under normal conditions (25°C) were 13.5 ± 1.08 and 15.5 ± 0.98 ng g FW^{-1}, respectively. After cold treatment for 24 h, the proline content in the 2-week-old seedlings of the wild type rice increased to 17.7 ± 1.12 ng g FW^{-1}, while that of the *OsCOIN* transgenic rice increased dramatically to 58.7 ± 1.76 ng g FW^{-1}. This result suggested that the proline level was dramatically increased by overexpression of *OsCOIN* after the cold treatment.

Discussion

In this report we have cloned and characterized *OsCOIN*, a novel cold-inducible gene in rice. Analysis of the deduced amino-acid sequence of the protein revealed that it had a RING finger domain (amino acid from 72 to 106), indicating that it belonged to a family of bZIP zinc finger proteins (Fig. 1b). OsCOIN protein is localized in both the nuclear and the cytoplasm (Fig. 2). In addition, we showed that the expression of *OsCOIN* could be induced by cold, ABA, salt and drought (Fig. 3). Furthermore we demonstrated that overexpression of *OsCOIN* in the transgenic rice lines significantly enhanced tolerance to cold, drought and salt treatment (Figs. 5, 6). Finally we showed that the expression of some of the known cold-responsive genes and the proline content of the cells were increased in the *OsCOIN* transgenic rice after the cold treatment (Fig. 7a, b).

Bioinformatic analysis showed that the OsCOIN protein had no classic mono-partite or bi-partite nuclear localization signal, but it is localized in both the nuclear and the cytoplasm membrane (Fig. 2). The reason is still unknown why the OsCOIN protein can enter the nucleus without an NLS. It is possible that the OsCOIN protein enters the nucleus by forming a heterodimer with another NLS-containing protein, just like OSISAP1, a zinc finger protein

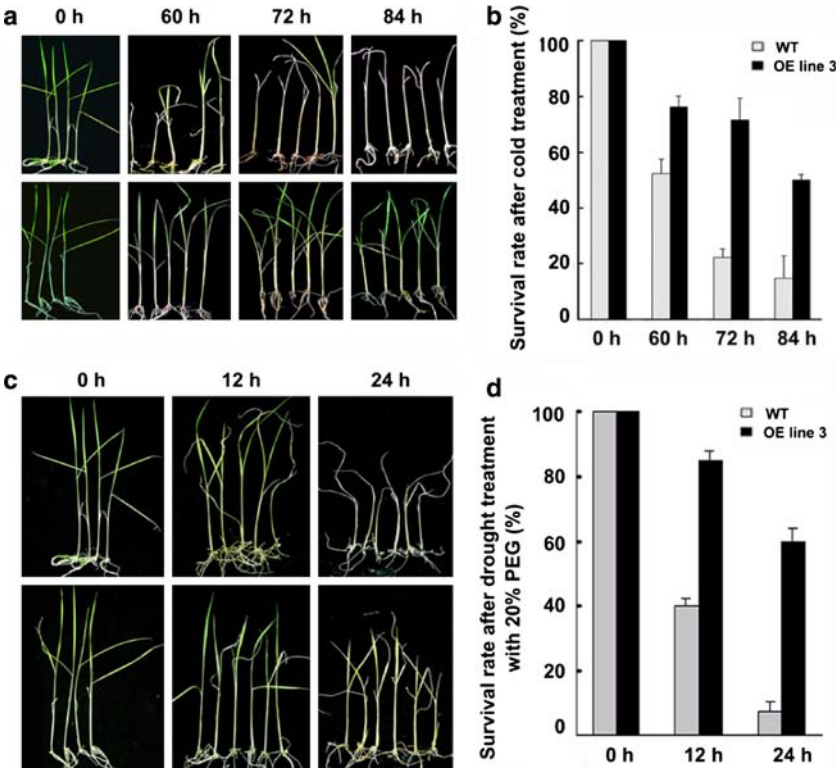

Fig. 5 Cold and drought tolerances of the *OsCOIN* transgenic rice. **a** Cold tolerance of the *OsCOIN* transgenic rice. Two-week-old rice seedlings of both wild type and *OsCOIN* transgenic rice were exposed to chilling temperature (4°C) for 60, 72, or 84 h, and then returned to the normal growth conditions in the greenhouse for 2 weeks to allow their recovery. **b** Statistic of the survival percentage after 4°C cold treatment ($n = 20$). **c** Drought tolerance of the *OsCOIN* transgenic rice. Two-week-old rice seedlings of both wild type and *OsCOIN* transgenic rice were submerged into 20% PEG6000 for 12 or 24 h, and then returned to the normal growth conditions in the greenhouse for 2 weeks to allow their recovery. **d** Statistic of the survival percentage after drought treatment with 20% PEG6000 ($n = 50$). Error bars represent S.E. in the mean of every treatment (ten-times repeated). Control: a control without cold treatment or a water control

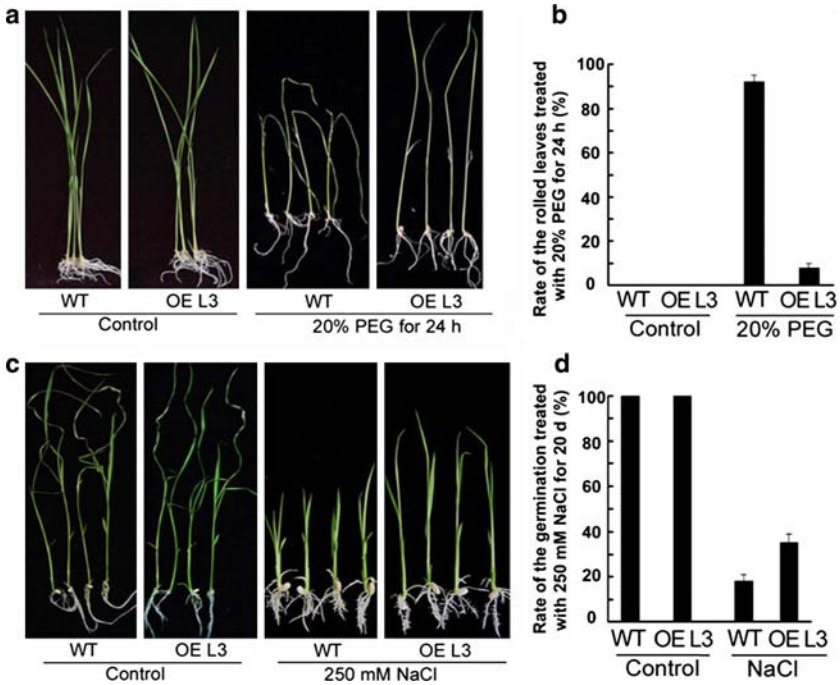

Fig. 6 Salt and drought tolerances of the *OsCOIN* transgenic rice. **a** The effect of drought treatment on 2-week-old seedlings of both wild type and *OsCOIN* transgenic rice ($n = 50$). **b** Statistic of the effect of drought treatment ($n = 50$) on 2-week-old rice seedlings. **c** The effect of 250 mM NaCl treatment on seed germination. **d** Statistic of the effect of 250 mM of NaCl treatment ($n = 50$) on seed germination. Error bars represent S.E. in the mean of every treatment (five-times repeated). Control: water controls or 1/2 MS without NaCl treatment on seed germination

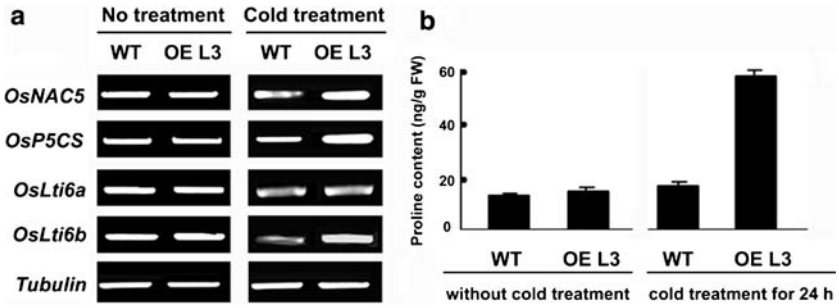

Fig. 7 Semiquantitative RT-PCR analysis of known cold-responsive genes and proline determination. Total RNAs from 2-week-old seedlings of both wild type and *OsCOIN* transgenic rice after cold treatment for 24 h were purified and reverse-transcribed. The resultant cDNAs were used as template for RT-PCR. The expression of *OsNAC6*, *OsP5CS*, *OsLti6a* and *OsLti6b* were analyzed with *tubulin* as a template control. PCR was performed at an annealing temperature of 56°C and 38 cycles were used for every gene and 28 cycles were used for *tubulin*. Experiments were repeated at least three times with similar results. No PCR band was observed in a water control. **b** Total RNAs from 2-week-old seedlings of both wild type and *OsCOIN* transgenic rice without cold treatment for 24 h were purified and reverse-transcribed. The resultant cDNAs were used as template for RT-PCR. PCR was performed at an annealing temperature of 56°C and 38 cycles were used for every gene and 28 cycles were used for *tubulin*. Experiments were repeated at least three times with similar results. No PCR band was observed in a water control. **c** Proline contents in the 2-week-old seedlings of WT and the *OsCOIN* transgenic rice plants with or without cold treatment for 24 h were measured as described by Bates et al. (1973). Error bars represent S.E. in the mean of proline contents (three-times repeated)

from rice (Mukhopadhyay et al. 2004). Another possibility is that a new kind of NLS is involved in the nuclear localization of the OsCOIN protein. New experiments will be underway to determine this possibility.

Some studies showed that the cold-response pathway was ABA-dependent (Hur et al. 2004; Morsy et al. 2005; Ohnishi et al. 2005). ABA level increased in response to low temperature and a set of cold-responsive genes such as *OsLti6*a/b were responsive to exogenous application of ABA. Thus, the expression of these cold-responsive genes was due, at least in part, to elevated levels of ABA. Nevertheless, recent effort has proved that cold-induced gene expression can also proceed through at least one ABA-independent pathway (Chen et al. 2003). The expression of *OsCOIN* was induced by exogenous ABA suggesting that the expression of the *OsCOIN* is ABA-dependent in rice (Fig. 3d).

It is very interesting that the expression of *OsP5CS* was increased 2.2-fold in our *OsCOIN* transgenic rice compared with the wild type rice (Fig. 7a). OsP5CS, an enzyme (delta1-pyrroline-5-carboxylate synthetase) induced by high salt, dehydration, ABA and cold treatments (Igarashi et al. 1997; Yamada et al. 2005), was involved in the biosynthesis of proline. Proline has been reported to play roles in protecting enzymes from denaturation (Nikolopoulos and Manetas 1991), stabilizing the machinery of protein synthesis (Kadpel and Rao 1985), regulating the cytosolic acidity (Venekamp et al. 1989), increasing water-binding capacity (Schobert and Tschesche 1978), and acting as a reservoir of carbon and nitrogen source (Fukutaku and Yamada 1984). Our results showed that the levels of proline in the *OsCOIN* transgenic rice were increased over threefold after 4°C cold treatment for 24 h compared with the levels of proline in the WT rice, although they remained at about the same level in both the *OsCOIN* transgenic rice and WT rice in the absence of any stress treatments (Fig. 7b). Therefore, our *OsCOIN* transgenic rice increased the tolerance to cold, salt, and drought at least partially by increasing the concentration of proline in the cells.

Previously the two closely related genes, *OsLti6a* and *OsLti6b*, have been shown to exhibit tissue-specific differential expression. *OsLti6a* showed high expression only in shoots in rice seedlings under cold stress and *OsLti6b*, in both shoots and roots. Both are involved in preserving the integrity of the plasma membrane (Morsy et al. 2005). In the 2-week-old seedlings of our *OsCOIN* transgenic rice lines the expression of *OsLti6b* is increased during cold treatment while that of *OsLti6a* remains about the same (Fig. 7a).

Conclusion

In this report we cloned and characterized *OsCOIN*, a novel cold-inducible gene in rice. OsCOIN, a putative RING finger protein, is localized in both nuclear and cytoplasm membrane. *OsCOIN* is expressed in all rice organs and strongly induced by low temperature, ABA, salt and drought. Overexpression of *OsCOIN* in transgenic rice significantly enhances the proline content of the cells and tolerance to cold, salt and drought treatment. Our data supported a hypothesis that OsCOIN enhances ABA-dependent tolerance to cold, salt and drought by increasing the proline level in the cells.

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