Identification and characterization of a stress-inducible gene *OsNLI-IF* enhancing drought tolerance in transgenic tobacco

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Plants respond to the adverse environment by activating a series of stress-inducible genes, including genes encoding transcription factors. The expression of these genes is regulated by a core DNA sequence contained in their promoter region called *cis*-acting element. The promoter region of several stress-responsive genes contains several stress-regulated *cis*-acting elements such as dehydration-responsive element, C-repeat, low-temperature-responsive element, NAC recognition sequence and ZFHD recognition sequence. In this study we isolated a cDNA for a transcription factor named nuclear LIM interactor-interacting factor from rice cDNA library by yeast one-hybrid screening using two target sequences of 50 nucleotides derived from two stress-inducible promoters, *JRC0528* and *JRC0332*, of cold-inducible genes *OsZF1* and *OsNAC6* respectively, as baits. The NLI-IF protein showed both DNA-binding and transcriptional activities in yeast experiments. Expression of *OsNLI-IF* was found to be induced by cold, heat, salt and drought stresses. The *OsNLI-IF* gene overexpressing transgenic tobacco plants showed improvement in drought tolerance. The present study emphasizes that *OsNLI-IF* could be useful for development of drought-tolerant transgenic crop plants.

Keywords: Rice, stress-inducible gene, transcription factor, transgenic tobacco.

PLANT growth is naturally affected by various environmental stresses such as drought, high salt, low and high temperature, and biotic stresses. In order to survive under extreme environmental conditions, expression of a variety of genes are induced in plant cells, which consequently leads to physiological and biochemical responses that increase stress tolerance of plants[1–3]. The products of these genes have been demonstrated to not only protect cells from stresses by the production of important metabolic proteins (functional proteins), but they also regulate the genes for signal transduction and gene expression in the stress responses (regulatory proteins)[1,4–6]. Transcription factors belong to the regulatory gene families and play an essential role in stress responses. Studies have demonstrated that altering the expression of certain transcription factors can greatly affect plant stress tolerance[7]. These transcription factors regulate the expression of their target genes by binding to the cognate *cis*-acting elements on the promoter region.

In *Arabidopsis thaliana*, *cis*-elements and corresponding binding proteins which contain distinct types of DNA-binding domains, such as APETALA2/Ethylene Responsive Factor (AP2/ERF), basic leucine zipper, homeodomain leucine zipper (HD-ZIP), MYB, MYC and several classes of zinc finger domains, have been implicated in plant stress responses because their expression is induced or repressed under different stress conditions[8,9]. The novel stress-inducible gene expression regulatory *cis*-acting element, which was first identified from the promoter of *RD29A* gene is 9 bp sequence TACCGACAT, known as the dehydration-responsive element (DRE)[10]. Similar motifs, TGGCCGAC and CCGAC, found in the promoter regions of cold-inducible genes *COD15a* and *BN115* were termed as C-repeat (CRT) and low-temperature-responsive element (LTRE) respectively[11,12]. Many cDNAs encoding ERF/AP2 type DRE- and CRT-binding proteins, including C-repeat binding factor (*CBF*) and DRE binding (*DREB*) protein, have been isolated using yeast one-hybrid system. There are 145 distinct genes encoding the ERF/AP2-type proteins in *Arabidopsis*, and these proteins have been classified into five groups, APETALA2 (AP2) subfamily, related-to-ABI3/VP1 (RAV) subfamily, DREB subfamily, ERF subfamily, and a specific gene *AL079349*, based on the similarity of their ERF/AP2 DNA binding domains[13]. The proteins of the DREB subfamily have been further divided into six groups, among which DREB1 (A-1) and DREB2 (A-2) were the two largest subgroups. Studies on transgenic plants suggested that DREB1 and DREB2 proteins are probably major transcription factors that function in cold,

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high salinity and drought-inducible gene expression in *Arabidopsis*[13–15].

Another *cis*-acting element, namely CATGTG identified in the promoter region of *erd1* gene, has an essential role in the regulation of gene expression during dehydration. This *cis*-acting element is recognized by proteins belonging to the largest plant transcription factor family NAC (NAM, ATAF and CUC) and therefore called NAC recognition sequence (NACRS). Previous studies have demonstrated that genes in the NAC family regulate not only a wide range of developmental processes, but also biotic and abiotic stress responses in plants. In transgenic rice, the *OsNAC6/SNAC2*, *OsNAC045* and *OsNAC10* genes were found to enhance drought and salt tolerance[7,16,17], and *SNAC1* increased grain yield (21–34%) under drought stress[18]. Using NACRS *cis*-acting element for yeast one-hybrid screening, Tran *et al.*[8] isolated three cDNAs encoding ANAC019, ANAC055 and ANAC072 transcription factors that increased drought stress tolerance of transgenic plants. In other study, a cDNA encoding the zinc finger homeodomain 1 (ZFHD1) transcriptional activator that specifically binds to the 62 bp ZFHD recognition sequence containing the *cis*-acting element CACTAAATTGTCAC in the promoter region of *erd1* was isolated. It was identified as a stress response-related transcription factor whose overexpression in transgenic plants enhanced drought tolerance but reduced the size of rosette plants and caused yellowing in some of the rosette leaves. However, the co-overexpression of the *ZFHD1* and *NAC* genes restored the morphological phenotype of the transgenic *Arabidopsis* plants and enhanced expression of the *erd1* gene to a near wild-type state[19].

In order to find a new stress-responsive *cis*-acting element, we analysed a variety of stress-inducible rice genes previously reported by Rabbani *et al.*[20]. We identified two motifs, CCTCCTCC and CTCCAC, which occur in the promoter region of several genes that may act as *cis*-acting elements in the regulation of gene expression. Interestingly, two cold-inducible genes *OsZF1* (AC090713.6) and *OsNAC6* (AF254558.1), named *JRC0332* and *JRC0528* respectively, contain both these motifs in their promoter regions. In this study, we used two 50-bp artificially synthesized sequences based on the nucleotide sequences of promoters *JRC0332* and *JRC0528* containing both hypothetical motifs as baits in yeast one-hybrid assay in order to screen rice high-salt and drought cDNA library. We identified a cDNA clone encoding a protein (transcription factor) binding to both target sequences, called *OsNLI-IF* (Nuclear LIM interactor-interacting factor). The expression profiles of *OsNLI-IF* under various stress treatments and its transcriptional activity were also studied. Additionally, DNA-binding and transcriptional activities of NIL-IF protein were supported via yeast experiments. In order to study the role of *OsNLI-IF* in abiotic stress, the performance of transgenic tobacco plants overexpressing *OsNLI-IF* was analysed.

Materials and methods

Plant materials, growth conditions and stress treatments

Seeds of rice Nipponbare (*Oryza sativa* L. ssp. *japonica*) were embedded in water at 37°C for two days and the seedlings were then hydroponically grown in MS solution at 28°C for three weeks. The three-leaf-old plants were subjected to different treatments as described previously[21]. The plants were transferred from the basal nutrient solution to nutrient solution containing 250 mM NaCl (salt treatment), and 20% polyethylene glycol (PEG) (drought treatment). For cold or heat treatment, plants were transferred to and kept at 4°C or 42°C respectively. For dehydration treatment, plants were exposed to air on tissue papers put in a box hood. Whole plants were harvested at different time points as shown in Figure 1 and frozen immediately in liquid nitrogen. Total RNA was isolated from stress-treated plant materials using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol.

Rice high-salt and drought cDNA library construction

mRNA was purified from total RNA by biotinylated-oligo (dT) probe and streptavidin paramagnetic particles (Promega), according to the manufacturer's protocol. Five microgram of mixed poly $(A)^+$ RNA population from a variety of conditions, including treatment with 250 mM NaCl, 20% PEG, cold (4°C) and heat (42°C) was used for the preparation of HybriZAP®-2.1 cDNA libraries (Stratagene) according to the supplied manual, with a minor modification. Instead of the suggested sepharose CL-2B column which demands radioactive materials, sephadex 400 column was used for cDNA size fractionation. Aliquots of the amplified HybriZAP®-2.1 libraries were stored in 7% (v/v) DMSO at –80°C until use.

Generation of yeast reporter strains

The target–reporter constructs were prepared by the cloning tandems containing four repeats of two novel *cis*-elements derived from the stress-inducible promoters *JRC0332* and *JRC0528* into pHISi-1 and pLacZi vector (Clontech, Palo Alto, CA). Integration of the reporter constructs into yeast (YM4271) genome was performed as described in the Yeast Protocol Handbook (Clontech). The background expression of reporter genes in yeast colonies with integrated target-pLacZi and pHISi-1 constructs (called Y0332 and Y0528) was tested according to the supplied MATCHMAKER One-Hybrid System User Manual (Clontech). The yeast strains with the lower background level of *HIS3* and *lacZ* were used in the one-hybrid cDNA library screening.

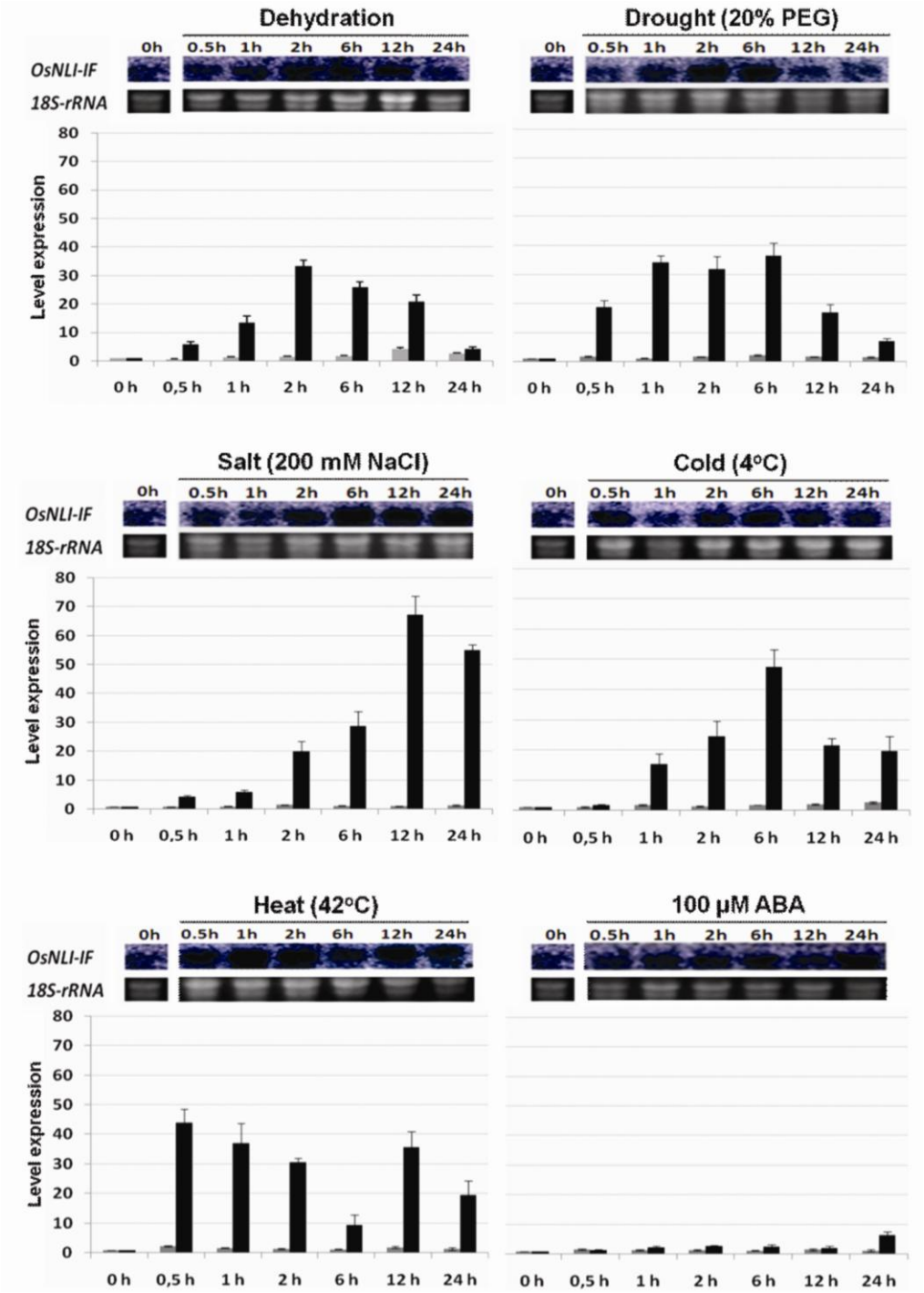

Figure 1. Regulation of *OsNLI-IF* expression by different stresses. Expression of *OsNLI-IF* was detected in shoot (grey column) and root (black column) tissue by real time RT-PCR (top) and RNA-gel blot assay (bottom) after treatments of dehydration (dried on air), cold (4°C), heat (42°C), salt (200 mM NaCl), drought (20% PEG) and hormone (100 µM ABA) for 0 (as a control), 0.5, 1, 2, 3, 6, 12 and 24 h. Values shown in the graphs are mean of data taken from three replicated experiments.

Screening of stress-treated rice cDNA libraries

Approximately 3.1×10^6 yeast transformants were screened using 20 µg of AD-cDNA libraries as described in MATCHMAKER One-Hybrid System User Manual (Clontech). The cDNA isolation, sub-cloning and sequencing of the positive clones were performed as described previously[14]. Positive cDNA clones were isolated by PCR with forward primer 5′-GCACAGTTGAAGTG-AACTTGC-3′ and reverse primer 5′-AGGGATGTTTA-

ATACCACTAC-3′ and ligated in pGEM-T Easy vector (Promega). Nucleotide sequence identity was analysed using BLAST program (GenBank, NCBI).

Northern blot analysis

RNA gel-blot analyses were carried out as described previously[22]. Specific DNA fragments of full-length *OsNLI-IF* cDNA and 18S rRNA (as a control) labelled with [α^{32}P]-dCTP were used as probes for hybridization. Total RNA was separated on 1.2% formaldehyde–MOPS agarose gels and blotted onto Hybond-N^+ membranes (Amersham Biosciences). After hybridization performed at 65°C, blots were washed twice in 2X SSC and 0.1% SDS for 20 min at 65°C and once in 1X SSC and 0.1% SDS.

Real-time RT-PCR analysis

Real-time RT-PCR was performed on the Applied Biosystems 7500 real-time PCR System using *OsNLI-IF*-specific primers (forward 5′-TTCATTCGACCACACAG-3′ and reverse 5′-TGGATCCAAGATGTCAAGC-3′). *Actin* gene was used as an internal reference gene for calculating relative transcript levels[23]. Optimized real-time amplification efficiency[24] of target and reference genes was 2.0.

Transcriptional and DNA-binding activation analysis in yeast

The DNA fragment containing full ORF of *OsNLI-IF* was amplified from the cDNA library with specific primers (forward 5′-GAATTCATGCCAGCACTGAGGATG-3′ and reverse 5′-CTCGAGTTATTGGAAAATCTCAGC-3′; *Eco*RI and *Xho*I sites are underlined). The amplified fragments were first cloned into pGEM-T Easy vector (Promega) and verified by sequencing.

For DNA-binding activation analysis, the DNA fragment containing full ORF of the novel gene was released by digestion with *Eco*RI/*Xho*I and fused in frame with GAL4 DNA-acting domain in pAD-GAL4 2.1 (Figure 2 *b*). This construct was transformed into yeast reporter strain YM4271 integrated target-pLacZi and pHISi-1, which were previously ligated to four tandem copies of target sequence (NACRS or JRC0332 or JRC0528 fragment; Figure 2 *a*). pAD-GAL4 and pAD/*OsNAC6* vectors were used as the negative and positive controls in this experiment. The DNA-binding activity of the candidate was evaluated based on the expression of reporter genes *HIS3* and *lacZ* as described in the Yeast Protocol Handbook (Clontech, USA).

For transcriptional activation analysis, *OsNLI-IF* cDNA fragment was cut out from recombinant cloning pGEM-T by digestion with *Eco*RI and ligated into the yeast-expression vector YepGAP[19]. The construct YepGAP/NLI-IF and YepGAP (as a negative control) were then transformed separately into YM4271 reporter yeast strain integrated target-pLacZi and pHISi-1 containing four tandem copies of JRC0332 sequence and wild-type YM4271 yeast strain. The transcriptional activity was evaluated based on the expression of reporter genes *HIS3* and *lacZ* as described in Yeast Protocol Handbook (Clontech).

Constructs and generation of transgenic plants

To generate the *OsNLI-IF* over-expression construct, the pGEM : *OsNLI-IF* plasmid was digested by *Eco*RI to release the 1.3 kb *OsNLI-IF* cDNA and the fragment was sub-cloned into pRT101. Subsequently, the *35S : OsNLI-IF : Nos-T* overexpression construct was cut out by digestion with *Hin*dIII and insert into plant expression pCAMBIA1301 vector (Figure 3 *a*). The resulting construct was electroporated into *Agrobacterium tumefaciens* LBA4404, which was used to transform tobacco using the method described by Horsch *et al.*[25]. The empty pCAMBIA1301 vector was used as the negative control. The hygromycin-resistant transgenic lines were confirmed by PCR analysis using *Nos* terminator-specific primer (forward: 5′-AGACCGGCAACAGGATTCAA-3′) and *OsNLI-IF* gene-specific primer (reverse: 5′-CGTTATTTCCGGGAGTCA-3′), and Western blot assay using anti-NLI-IF (as primary antibody) and anti-mouse IgG (as secondary antibody).

Production of polyclonal antibody and Western blot analysis

Anti-NLI-IF antiserum was raised in mouse according to the method of Amero *et al.*[26], using the recombinant protein NLI-IF samples, which were expressed and purified in our previous study[27]. Ig-G polyclonal antibody was purified using Pierce protein A columns, as described in the manufacturer's instructions (Thermo Scientific).

For Western blot analysis, the total proteins were extracted from the unstressed tissue samples of transgenic lines as well as wild-type plants, using a protein extraction buffer (pH 8.0, 100 mM Tris-HCl, 1 mM PMSF, β-mercaptoethanol, sodium sulphate, 2% PVP) and then an equal amount of each concentrated protein sample was run on 12% SDS-PAGE according to a previously described method[28,29] and transferred to a PVDF membrane. The membranes were blocked for 1 h with PBS-BSA 1%. The blots were probed with purified anti-NLI-IF antibody for 1 h at 37°C. Bound antibodies were detected using rabbit alkaline phosphatase (AP)-conjugated anti-mouse IgG antibodies (Sigma) and an AP conjugate substrate Kit (Bio-Rad), according to the manufacturer's instructions.

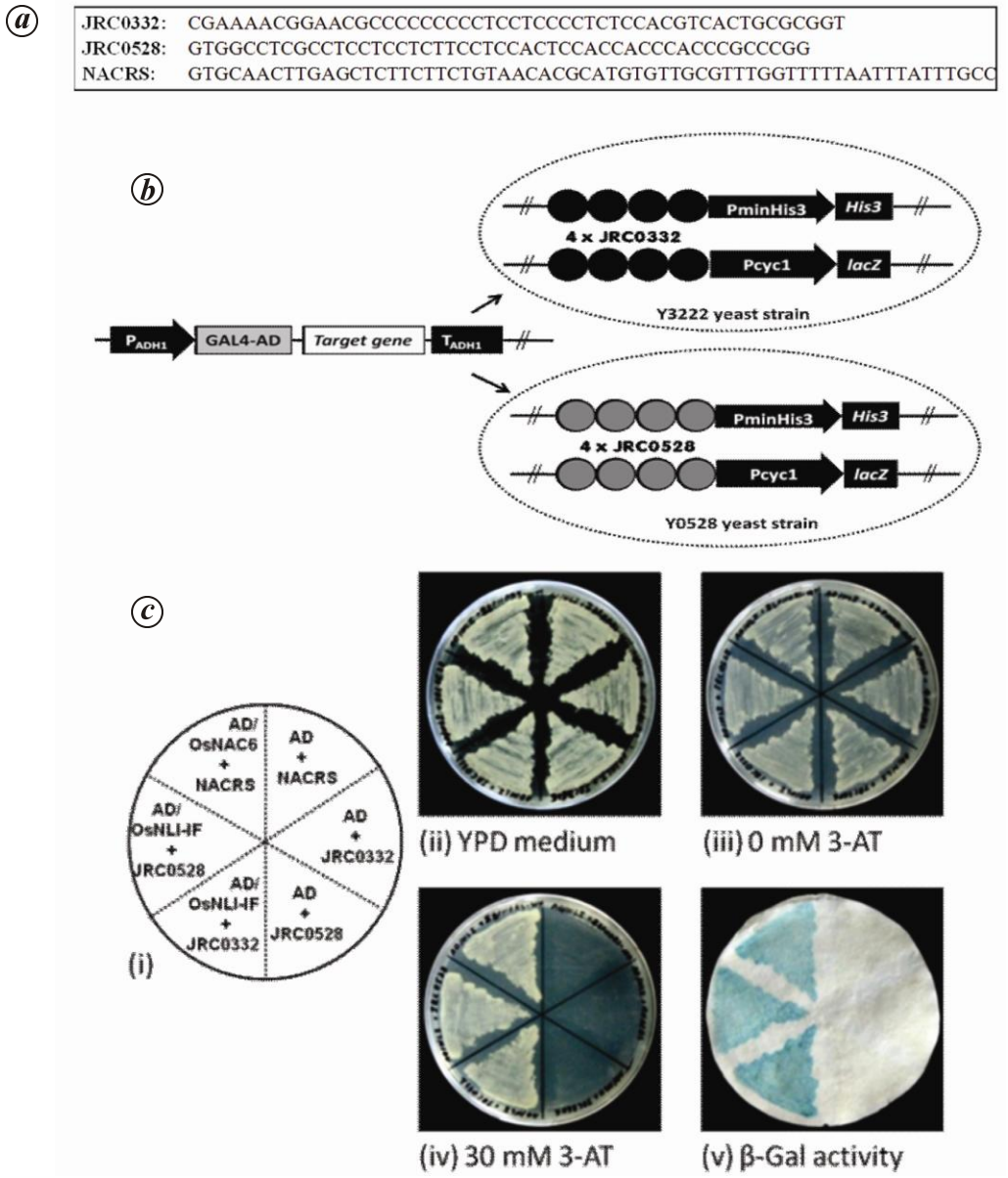

Figure 2. Isolation of cDNA-encoding NLI-IF protein using yeast one-hybrid system. ***a***, Sequence of three target DNA fragments. ***b***, Schematic drawing of pAD-GAL4 vector fused with cDNA clones and the two yeast reporter strains carrying dual reporter genes *HIS3* and *lacZ* under the control of novel *cis*-acting element JRC0332 or JRC0528. ***c***, The full OsNLI-IF protein fused to GAL4-acting domain in pAD-GAL4 vector and then introduced into three yeast reporter strains carrying the reporter genes driven by the different promoters constructed with four tandemly repeated fragments NACRS or JRC0332 or JRC0528. (i) Template showing the organization of yeast experiment. (ii) Phenotypes on a YPD plate. (iii) Yeast growing on a SD/Leu-/His-/Ura-medium without 3-AT competitor. (iv) Yeast growing on a SD/Leu-/His-/Ura-medium containing 30 mM 3-AT. (v) β-Galactosidase filter lift assay. Yeast cells carrying the reporter genes driven by NACRS and transformed pAD-GAL4 vector containing full ORF of *OsNAC6* gene were used as positive control.

Stress tolerance assay

Analyses of stress tolerance were done according to the method of Tran *et al.*[8] and Dubouzet *et al.*[30] methods. In brief, T1 seeds were grown in petri dishes containing selective agar germination medium for two weeks and then transferred to 8 cm vermiculite pots, and grown for two more weeks. Four-week-old positive transgenic plants confirmed by PCR and Western blot assay and showing similar phenotype to wild-type plants were subjected to drought treatment. Drought stress was built up by keeping the plants without watering for four weeks, and then the dehydrated plants were rewatered for three days. After drought treatment, the number of plants that survived and continued to grow was counted.

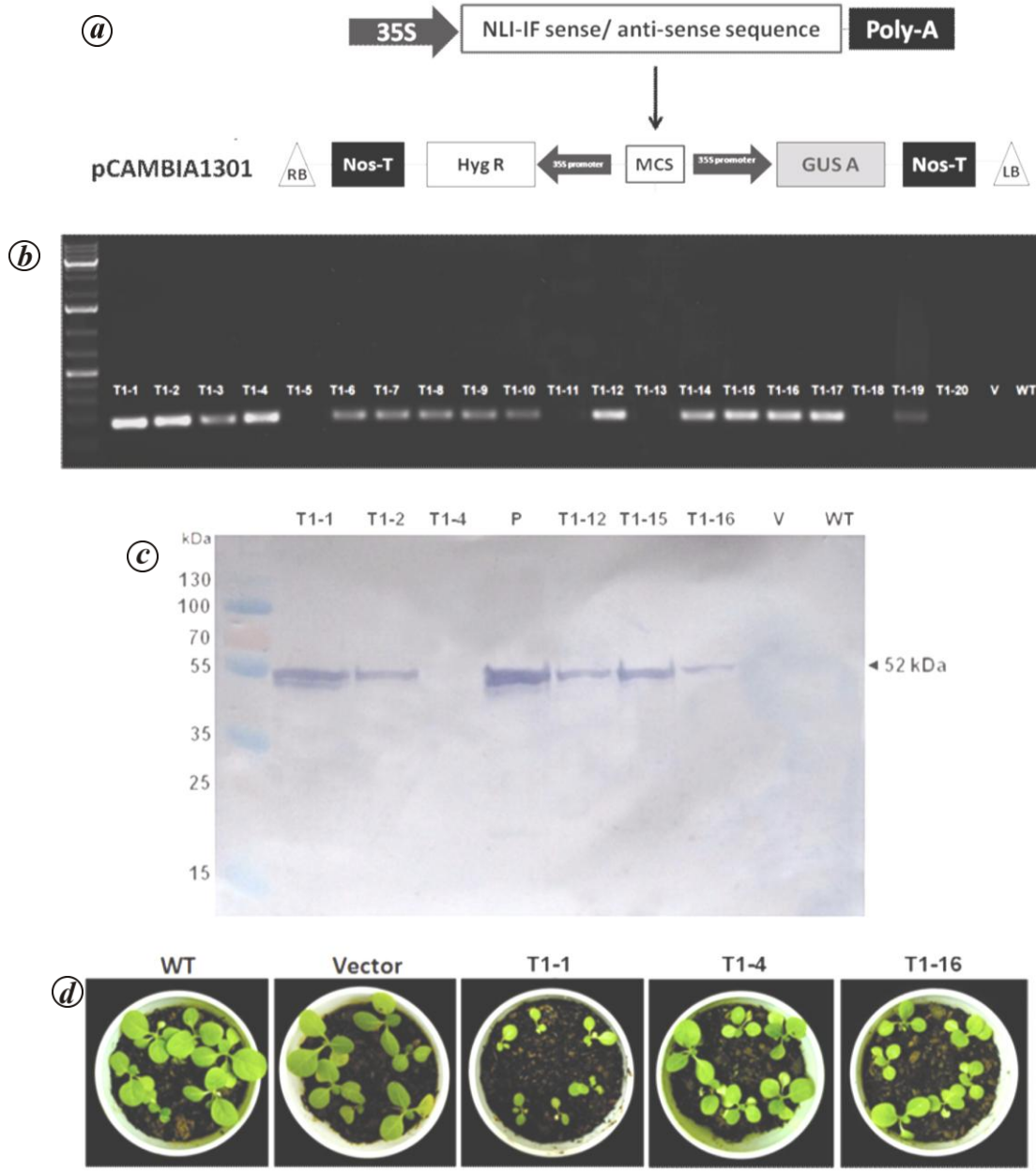

Figure 3. Molecular and morphological analysis of *OsNLI-IF* overexpressing transgenic tobacco plants. ***a***, Schematic drawing of plant expression vector used in research. ***b***, PCR analysis using *OsNLI-IF* and *Nos* terminator-specific primers. ***c***, Western blot analysis of transgenic and wild-type plants using anti-OsNLI-IF polyclonal antibody; recombinant OsNLI-IF protein was used as positive control. ***d***, Wild-type plants, pCAMBIA1301 transgenic plants and *35S : OsNLI-IF* transgenic plants (lanes T1-1, T1-4 and T1-16) in soil pots under normal growth condition. T1 *OsNLI-IF* transgenic lines are numbered from T1-1 to T1-20; WT, Wild-type plants; V/Vector, pCAMBIA1301 transgenic plants; P, Recombinant OsNLI-IF protein.

Results

Isolation of a cDNA encoding a novel protein that binds to the 50 bp DNA fragments derived from JRC0332 and JRC0528 promoters

To isolate the cDNA-encoding protein of interest, yeast one-hybrid screening was performed. We used two 50-bp DNA fragments derived from two promoters, *JRC0332* and *JRC0528* (which regulate the expression of cold-inducible genes *OsZF1* and *OsNAC6* respectively) as baits for isolation of transcription factor (see Figure S1 *a*, Supplementary information online). Vectors containing reporter genes driven by four repeats of *JRC0332* and *JRC0528* were separately transformed into YM4271 yeast cell (and then called Y0332 and Y0528 strains respectively). The results of tests for *HIS3* and *lacZ* background expression using control assay showed that: (i) both

Table 1. Results of yeast one-hybrid screening using target sequences derived from two stress-inducible promoters, *JRC0032* and *JRC0528*

JRC0032	JRC0528
Five nuclear LIM interactor-interacting factors	Three nuclear LIM interactor-interacting factors
One zinc finger protein	One typical-P-type R2R3 Myb protein
One zinc finger (C3HC4-type ring finger) protein	One cold-induced protein
One glycine-rich RNA binding protein	One EIF 4D translation initiation factor
One 60S ribosomal protein	One putative dehydrogenase
One metallothionein-like protein	One glycine-rich RNA-binding protein
One ribulose–bisphosphate carboxylase	One RNA-binding protein
One fatty acid (NAD-binding domain)	One Systeine synthase
One lipid transfer protein	One putative acyl carier protein
One hypothetical protein	Three hypothetical proteins
Four vector sequences	Five vector sequences

Y0332 and Y0528 transcribe the *HIS3* gene at basal levels; and (ii) they form white colonies on filter paper that was pre-incubated in X-Gal solution for 60 min. In order to screen cDNAs, we separately transformed the two target reporter strains Y0332 and Y0528 with pAD-GAL4 cDNA library, which was constructed from a mixture of cDNA fragments of mRNAs prepared from rice treated with high salt and drought (Figure S1 *b*, see Supplementary information online). Nineteen yeast clones resistant to 10 mM 3-AT and forming blue colonies in β-galatosidase assay were isolated from the library using yeast reporter strain Y0528. On using yeast reporter strain Y0332, we isolated 18 positive clones. All the positive cDNA clones were sub-cloned into pGEM-T vector and submitted for sequencing (Table 1). We identified eight homologous cDNA clones encoding a protein, named nuclear LIM interactor-interacting factor (NLI-IF), which showed DNA-binding activity in both the screening experiments. To confirm whether this protein specifically binds to both target sequences JRC0332 and JRC0528, the full length ORF of *OsNLI-IF* was sub-cloned into pAD-GAL4 vector and separately introduced into Y0332 and Y0528 yeast reporter strains. Another yeast reporter strain carrying reporter genes driven by a four-time tandemly repeated 63-bp DNA fragment of *erd1* promoter containing the CATGTG motif (NACRS), called Y-NACRS strain, was used as a negative control[8]. Either Y0332 or Y0528 yeast reporter cells transformed with the plasmid containing *OsNLI-IF* gene grew on the His-lacking medium in the presence of 30 mM 3-AT and induced the *lacZ* activity, while control yeast cells did not show the expression of both reporter genes *HIS3* and *lacZ* (Figure S1 *c*, see Supplementary information online). These results indicate that isolated cDNA encoding NLI-IF specifically bound to both target sequences JRC0332 and JRC0528.

Expression profile of the OsNLI-IF gene

The expression pattern of *OsNLI-IF* gene was analysed using RNA-gel blot assay and real-time RT-PCR. We performed a time course experiment using five kinds of treatment (Figure 1). Three-week-old rice seedlings were dehydrated (dehydration), transferred to 42°C (heat) and 4°C (cold), hydroponic growth in 250 mM NaCl (salt), 20% PEG (drought) and 100 µM ABA (hormone treatment). The real-time PCR results revealed that *OsNLI*-IF almost functions in root tissues, but not in shoots during stress treatment. The amount of *OsNLI-IF* mRNA did not increase in ABA-treated plants; it was significantly accumulated within 2 h in plants subjected to cold and salt treatments and peaked at 6 h and 12 h respectively, after treatment. Under dehydration treatment, *OsNLI-IF* was quickly induced, peaked at 2 h after treatment and then decreased. On heat treatment, a rapid and high level of accumulation of *OsNLI-IF* mRNA was detected after 30 min stress exposure; the maximum level was observed at 1 h and the decline of transcript level started 2 h after treatment. However, after 6 h, the expression of *OsNLI-IF* increased again until over 12 h. The accumulation of *OsNLI-IF* mRNA under drought and cold conditions was slower than under heat condition, but also reached a peak at 6 h. However, the *OsNLI-IF* expression levels under drought treatment decreased rapidly to basal line after 12 h of stress. In contrast, under heat, cold, dehydration and especially salt treatment, high expression of *OsNLI-IF* was continuously observed over 24 h. The highest expression of *OsNLI-IF* was observed at 12 h after treatment when plants were subjected to salt stress. These results demonstrate that the expression of *OsNLI-IF* was induced in root tissues by almost all stress treatments, including drought, dehydration, salt, cold and heat stress.

The OsNLI-IF protein has transcriptional activation

To validate that the *OsNLI-IF* functions as a transcriptional activator, we used yeast one-hybrid assay. The full ORF of *OsNLI-IF* was ligated into the yeast expression vector YepGAP and then the resulting construct was transformed into yeast reporter strain integrated target-pLacZi and pHISi-1 containing four tandemly repeated fragments JRC0332 (Y0332 strain) and wild-type yeast

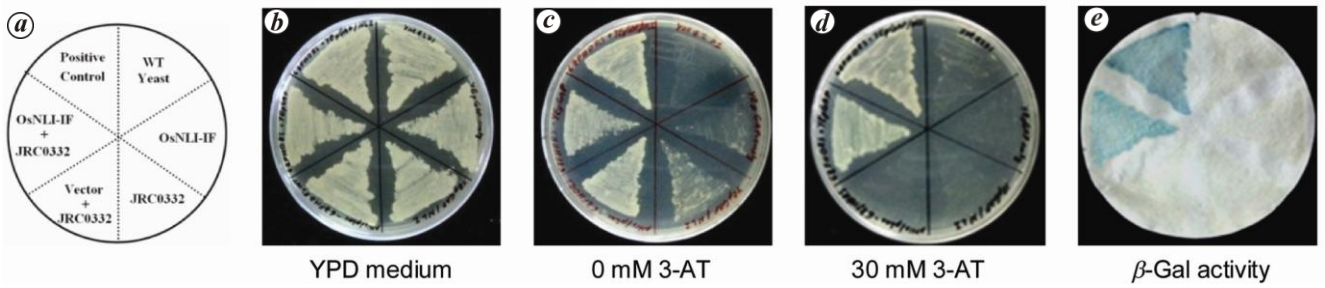

Figure 4. Transcriptional activity of *OsNLI-IF* in yeast cells. The full-length ORF of *OsNLI-IF* was cloned into YepGAP vector and then introduced into the yeast reporter strain Y0332 carrying the reporter genes driven by the promoter constructed with four tandemly repeated fragment JRC0332 (OsNLI-IF + JRC0332). Yeast cells YM4271 (WT Yeast), YM4271 transformed YepGAP/OcNLI-IF vector (OsNLI-IF), Y0332 (JRC0332) and Y0332 transformed YepGAP vector (Vector + JRC0332) were used as the negative controls. For positive control in this experiment, yeast cells Y0332 carrying the OsNLI-IF-cDNA-fused pAD-GAL4 vector were used. ***a***, Template showing the organization of yeast experiment; ***b***, Showing phenotypes on a YPD plate; ***c***, Yeast growing on a SD/Leu-/His-/Ura-medium without 3-AT competitor; ***d***, Yeast growing on a SD/Leu-/His-/Ura-medium containing 30 mM 3-AT; ***e***, *β*-galactosidase filter lift assay.

strain (YM4271 strain). The YM4271 wild-type cells, Y0332 cells transformed with or without YepGAP empty vector were used as negative control in this experiment. Additionally, we also used yeast reporter strain Y-NACRS transformed vector pAD/*OsNAC6* as the positive control. As shown in Figure 4, all studied transformants grew well on YPD medium and the transformants containing adequate amount of constructs pHISi, pLacZi and YepGAP could grow on SD/Trp-/His-/Ura-medium. However, only the yeast reporter cells which were transformed with vector YepGAP/*OsNLI-IF* could grow on the selection medium in the presence of 30 mM 3-AT and showed the expression of reporter gene *lacZ* in *β*-galactosidase assay. These results indicate that *OsNLI-IF* functions as a transcriptional factor expressing both DNA-binding and transcriptional activity.

Overexpression of OsNLI-IF in transgenic plants related to stress tolerance

To characterize the *in vivo* function of OsNLI-IF protein, transgenic tobacco plants overexpressing *OsNLI-IF* were generated by *Agrobacterium*-mediated transformation. The *OsNLI-IF* cDNA was overexpressed by enhanced *CaMV 35S* promoter in tobacco[31]. After selection by growing on hygromycin MS medium, PCR test and Western blot analysis were employed to screen the expression level of *OsNLI-IF* for each of the 20 resulting T1 plants. As the control, plants introduced with empty expression construct (control transgenic plants) were analysed together with wild-type and *OsNLI-IF* transgenic plants. Among 20 tested transgenic lines, 16 showed the presence of *OsNLI-IF* in PCR test (Figure 3 *b*) and five lines showed expression of transgene via Western blot analysis using anti-NLI-IF polyclonal antibody (Figure 3 *c*). As shown in Figure 3 *d*, the transgenic lines accumulating OsNLI-IF protein at the highest level (line T1-1) exhibited growth retardation and a significantly delayed bolting time. In contrast, the transgenic plants overexpressing *OsNLI-IF* at the lowest level (line T1-16) showed insignificantly different phenotype in comparison with wild-type and control transgenic plants. These results indicate that the degree of the growth retardation and dwarfed phenotype of these plants seems to be correlated with the overexpression level of the transgene and the higher level of OsNLI-IF protein accumulation resulted in more dwarf phenotype of plants.

Notably, drought tolerance analysis indicated that the survival rates of transgenic plants were significantly higher than those of wild-type plants. After recovering from drought treatment, the survival percentage of wild-type plants was 16 (6/36), whereas that of transgenic lines T1-1 and T1-16 was 56 (27/37) and 75 (27/36) respectively (Figure 5). These results suggest that the overexpression of *OsNLI-IF* may confer drought tolerance to the transgenic tobacco plants.

Discussion

To date, several genes related to plant stress tolerance which encode transcription factors such as DREB, NAC, MYB and zinc finger protein have been identified[7,8,13,16]. Most of them were isolated using one-hybrid screening in yeast, a powerful method to rapidly identify heterologous transcription factors[8,14,19,32]. In this article, we report the results of isolation of a novel transcription factor from rice cDNA library, using two target sequences derived from the promoter sequences of two stress-inducible genes *OsNAC6* and *OsZF1*. OsNAC6 transcription factor that plays an important roles in both biotic and abiotic stress responses was previously identified[17,33]. A previous study on the promoter of *OsNAC6* (named *JRC0528* promoter) showed that it was induced by a variety of different stress treatments, including high salinity, drought, cold and ABA[19]. Our sequence analysis based on the MEME program revealed that the *JRC0528* promoter

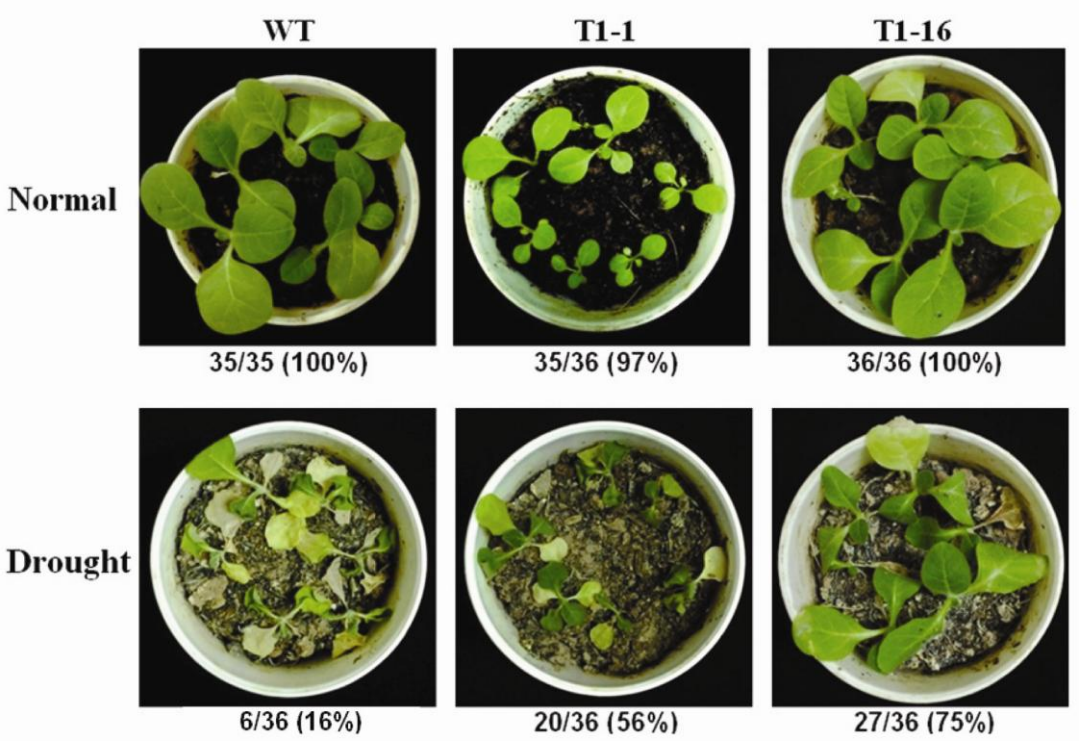

Figure 5. Effects of drought stress on transgenic tobacco plants overexpressing *OSNLI-IF*. Drought tolerance of wild-type (WT) and *OsNLI-IF* transgenic plants overexpressing transgene at high (lane T1-1) and low (lane T1-16) levels was analysed. Four-week-old plants were withheld from water for four weeks, and then the dehydrated plants were rewatered for three days. The number of plants surviving is indicated below each photograph.

contains two motifs, CCTCCTCC and CTCCAC, which seem to play the role of novel *cis*-acting elements. Interestingly, we also found the presence of these motifs in the promoter sequence of several cold-inducible genes, including *OsZF1* gene (named *JRC0332*), glutamate dehydrogenase-like protein encoding gene (*JRC2606*), basic/leucine zipper-protein encoding gene *lip19* (*JRC1317*) and an unknown protein encoding gene (*JRC0937*). In this study, we used two 50-bp sequences containing two predicted motifs which were extracted from promoters *JRC0528* and *JRC0332* as baits for yeast one-hybrid screening experiments and identified a novel transcription factor encoded by *OsNLI-IF* gene from the cDNA library of stress-treated rice.

NLI-IF protein is a nuclear protein whose function is little understood. However, several publications have reported the involvement of NLI-IF protein in neural development in mammals[34,35] or embryonic development in insect[36]. In addition, studies have also revealed that NLI-IF is located in the nucleus and functions as a transcriptional regulator by protein–protein interaction to regulate the expression of other genes[34–36]. However, the role of this protein in plants has yet to be established. Interestingly, in this study, NLI-IF showed DNA-binding activity by which the NLI-IF fusion protein containing AD-GAL4 domain could bind to the core sequence in the upstream promoter region of reporter genes and induce the expression of histidine and β-galactosidase (Figure 2). Furthermore, yeast experiments also revealed that OsNLI-IF harbours additional *in vivo* transcriptional activation domain (Figure 4). Although our experiments did not show the DNA-binding specificity of NLI-IF protein, based on the fact that OsNLI-IF could recognize the DNA motif in both JRC0332 and JRC0528 sequence, but not CATGTG motif in NACRS (shown in Figure 2) and DRE motif (data not shown here), we predict that the DNA motifs recognized by NLI-IF could be CCTCCTCC or CTCCAC. However, more experiments needed to further confirm the predictions[8,19].

Previous studies have shown that many transcription factors from different plants, though all classified into the same group, respond differently to stresses[37–40]. *ZmDBF1* shows a response to dehydration and sal[41]; *AhDREB1* plays a role in the salt- and drought-responsive pathways[42]; the transcripts of *GmDREBb* are induced by low temperature as well as salinity and drought[43]. Our Northern blot and real-time RT PCR analysis revealed that the expression of *OsNLI-IF* in rice was upregulated mostly in the roots by multiple stress signals, including salt, heat, cold, drought and dehydration (Figure 1). Additionally, our experiment also revealed that transcription of OsNLI-IF did not change when the plants were treated with ABA, which implied that OsNLI-IF probably participates in the ABA-independent stress signal transduction

pathway. This finding supports previous knowledge that there is variation of transcriptional regulation mechanism for stress-induced transcription factors, which demands further studies for a greater understanding of the same.

In order to verify the functional role of *OsNLI-IF* in abiotic stress tolerance, we analysed transgenic tobacco plants overexpressing *OsNLI-IF* controlled by constitutive promoter *35S*. Our experimental results showed that overexpression of *OsNLI-IF* causes obvious growth retardation of *35S : OsNLI-IF* transgenic plants in which the decrease in growth rate was correlated to accumulation of *OsNLI-IF* protein (Figure 3 *c* and *d*). The overexpression of some other stress-responsive genes, such as *OsNAC6/SNAC2*, *OsDREB1A*, *OsDREB1B*, *AtDREB1A* and *AtDREB1B* led to growth retardation of transgenic plants under normal conditions, which may finally cause significant reduction of potential yield[17,42,44–47]. Maruyama *et al.*[48] studied the mechanism of growth retardation of *35S : DREB1A* transgenic plants and found that the transgene upregulated some transcription factors whose expression represses photosynthesis and carbohydrate metabolism in transgenic plants.

We note here that drought tolerance of the transgenic lines is correlated to expression level of *OsNLI-IF* in stress tolerance assay. In previous reports, the overexpression of identified stress-responsive transcription factors either induced increased or decreased stress tolerance of transgenic plants. However, several reports[49,50] suggested that changes in the expression level of a transcription factor may lead to various degrees of sensitivity to different stresses, such as *XERICO* and *ABR1*. Similarly, our drought experiments showed that transgenic plants overexpressed *OsNLI-IF* at a low level, displaying a survival rate which is significantly higher than wild-type plants (75% versus 16%) and higher than transgenic plants accumulating *OsNLI-IF* at a high level (75% versus 56%; Figure 5). Although further studies are required to understand the exact regulatory functional mechanism of *OsNLI-IF* in drought response of plants, our findings suggest that it seems to be a novel transcriptional activator that may regulate plant drought tolerance via an ABA-independent pathway and may be useful in improving stress tolerance in plants.

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