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Genome-scale screening and molecular characterization of membrane-bound transcription factors in *Arabidopsis* and rice

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ABSTRACT

Controlled proteolytic activation of membrane-bound transcription factors (MTFs) is recently emerging as a versatile way of rapid transcriptional responses to environmental changes in plants. Here, we report genome-scale identification of putative MTFs in the *Arabidopsis* and rice genomes. The *Arabidopsis* and rice genomes have at least 85 and 45 MTFs, respectively, in virtually all major transcription factor families. Of particular interest is the NAC MTFs (designated NTLs): there are at least 18 NTLs in *Arabidopsis* and 5 NTL members (OsNTLs) in rice. While the full-size OsNTL forms are associated with the membranes, truncated forms lacking the transmembrane domains are detected exclusively in the nucleus. Furthermore, transcript levels of the *OsNTL* genes were elevated after treatments with abiotic stresses, supporting their roles in plant stress responses. We propose that membrane-mediated transcriptional control is a critical component of gene regulatory network that serves as an adaptive strategy under unfavorable growth conditions.

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Introduction

Transcription factors are critical components of transcriptional regulatory schemes controlling expression of genes in response to intrinsic and external signals. Their activities are coordinately regulated at multiple steps through transcriptional, posttranscriptional, translational, and posttranslational mechanisms [1–3]. The most recently emerging one is controlled proteolytic activation of membrane-anchored, dormant transcription factors. In this mechanism, the transcription factor protein is expressed as a membrane protein. Upon stimulation, the dormant form is activated through proteolytic cleavage, and the transcriptionally active form enters the nucleus, where it regulates expression of target genes [4,5].

In animals, the SREBP (*s*teroid *r*esponse *e*lement *b*inding *p*rotein) transcription factor is associated with the ER membranes [6–8]. When cells are depleted of cholesterol, the S1P (site-1 protease) and S2P (site-2 protease) proteases cleave the transcription factor in a tightly controlled manner [9–11].

Recently, several membrane-bound transcription factors (MTFs), belonging to the bZIP (basic leucine zipper) and NAC (NAM/ATAF1/2/CUC2) transcription factor families, have been identified in *Arabidopsis* [3,5]. The first reported MTF, AtbZIP60, possesses a bZIP domain and a single transmembrane (TM) motif. The *AtbZIP60* gene is induced by ER stress, which also induces proteolytic cleavage of the AtbZIP60 protein [12,13]. The *AtbZIP17* gene is induced by high salt, and the encoded protein is cleaved by an *Arabidopsis* S1P homolog, AtS1P [14].

Several MTFs belonging to the NAC transcription factor family have also been characterized in *Arabidopsis*. The NTM1 (*N*AC with *T*rans*m*embrane *M*otif *1*) transcription factor possesses a NAC domain in its N-terminal region and a TM motif in its far C-terminal region [15]. The active NTM1 form regulates cell division by inducing a subset of cyclin-dependent kinase inhibitor genes, *ICKs*/*KRPs* (*Interactors of Cdc2 kinase*/*Kip-related protein*). The *NTL8* (*NTM1 Like 8*) gene is mainly expressed in the imbibed seed and induced by high salinity and GA biosynthesis inhibitor, paclobutrazol [16]. It is notable that virtually all of the reported plant MTFs are closely related with plant responses to various abiotic stress conditions [3,5], supporting that controlled activation of the MTFs provides a way of rapid transcriptional responses to abrupt environmental changes in plants.

The rice genome contains approximately 140 NAC transcription factors, among which a few members have been functionally characterized [17]. The *SNAC1* (*stress-responsive NAC1*) and *SNAC2* genes are induced by abiotic stresses [18,19]. The *SNAC2* gene is related with salt tolerance. In contrast, the *OsNAC6* gene is induced by a variety of biotic and abiotic stresses, and *OsNAC6*-overexpressing, transgenic plants are resistant to drought, high salt, and to blast disease [20].

Recently, analysis of the protein structures of the NAC transcription factors in rice has shown that at least five NAC members are membrane-associated [3]. However, membrane association of the rice NAC transcription factors has not been confirmed yet. Furthermore,

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although it is estimated that a significant portion of plant transcription factors is membrane-associated [3], plant MTFs have not been systematically explored at the genomic levels.

In this study, we report genome-scale identification of plant MTFs in *Arabidopsis* and rice. We found that at least 85 and 45 transcription factors are membrane-associated in *Arabidopsis* and rice, respectively. Gene expression analysis revealed that they are responsive to diverse abiotic stresses. We therefore propose that membrane-mediated transcriptional regulation is a wide-spread, adaptive strategy that modulates plant responses to environmental stresses in the genomes of higher plants.

Results

Arabidopsis and rice genomes encode numerous MTFs

First-step screening OF MTFs was carried out using the ARAMEMNON membrane protein database, which includes putative membrane-associated proteins in the *Arabidopsis* and rice genomes and related analysis tools [21]. Transcription factors having TM motifs with hydrophobicity values of higher than 0.5 were subject to second-step screening according to the method of Kyte–Doolittle. Amino acid sequences of the *Arabidopsis* transcription factors were obtained from The *Arabidopsis* Information Resource (TAIR) database and those of rice transcription factors were extracted from the Rice Transcription Factor (DRTF) database [22]. The hydrophobic scales of the sequences were analyzed using the ConPred II program [23]. We selected putative MTFs which have hydrophobic scales of higher than 1.5. The final-step evaluation was carried out using the Gene Ontology database in the TAIR web site, from which total membrane proteins and total transcription factors are available. We finally chose those included in both the protein groups using a simplified visual basic program, which was operated through the EXCEL program as described in Fig. S1.

We found that the *Arabidopsis* genome contains at least 85 putative MTFs having protein sizes ranging from 115 (At2g13960) to 1595 (At5g12400) residues (Table 1), including the 13 NAC MTFs and 3 bZIP MTFs that have been previously reported. It was therefore estimated that approximately 4.5% (85/1900) of total transcription factors (http://datf.cbi.pku.edu.cn/) are membrane-associated in *Arabidopsis*. Although MTFs were identified in virtually all major transcription factor families, the relative frequencies were higher in some families. The relative frequency was highest in the NAC family. In this screening, we identified five additional NAC MTFs, indicating that at least 18 members among the approximately 110 NAC transcription factors are membrane-associated in *Arabidopsis*.

All the identified *Arabidopsis* MTFs, except for At5g35210 that has four TM motifs, have one or two TM motifs (Fig. 1). The locations of the TM motifs were also variable: among the 85 MTF members identified, 17 MTF members (20%) had the TM motifs in their N-terminal regions, and 61 MTF members (72%) had the TM motifs in their C-terminal regions. The TM motifs were also present in the central regions of 7 MTF members (8%), suggesting that membrane topologies of the MTFs and their activation mechanisms would accordingly be variable.

Membrane-associated proteins are divided into three different types, based on their membrane topologies. Type I and type II membrane proteins have a single TM motif, while type III membrane proteins have multiple TM motifs. The C-terminal of type I proteins is present in the cytoplasmic side, while the N-terminal of type II proteins is present in the cytoplasmic side. All of the NAC MTFs and SBP (squamosa promoter binding protein) MTFs are type II membrane proteins. In contrast, the PHD (plant homeodomain) and zinc finger MTFs contain both the type I and type II proteins.

It is notable that primary transcripts of some *MTF* genes are alternatively spliced and the alternatively spliced forms encode proteins lacking the TM motifs in *Arabidopsis* (Fig. S2). For example, a bHLH (basic helix-loop-helix) transcription factor (At1g59640) contains a single TM domain, but an alternatively spliced form (At1g59640.1) lacks

Table 1
Arabidopsis membrane-bound transcription factors (MTFs).

TF family	AGI code	Size (aa)	TF family	AGI code	Size (aa)	TF family	AGI code	Size (aa)
AP2	At1g64380	335	LOB	At3g47870	328	PHD	At5g26210	255
AT HOOK	At1g63470	378	MYB	At2g13960	115	PHD	At5g35210	1576
AT HOOK	At1g63480	361	MYB	At3g01140	388	RWP-RK	At4g35590	370
AT HOOK	At2g33620	351	MYB	At5g11510	798	RWP-RK	At4g38340	767
AT HOOK	At5g62260	404	MYB	At5g45420	309	SBP	At1g20980	1035
ATFII 55	At1g55300	203	NAC	At1g01010	429	SBP	At2g47070	881
AUX/IAA	At4g28640	302	NAC	At1g32870	528	SBP	At3g60030	927
B3	At2g24680	851	NAC	At1g33060	648	SBP	At5g18830	801
B3	At3g11580	230	NAC	At1g34180	564	SCARECROW	At1g07520	695
B3	At4g21550	721	NAC	At1g34190	557	SCARECROW	At2g29060	1336
bHLH	At1g59640	343	NAC	At1g65910	631	SPL16	At1g76580	809
bHLH	At1g62975	259	NAC	At2g27300	335	Tetratricopeptide	At4g37460	1052
bHLH	At1g71200	255	NAC	At3g10500	549	WHIRLY	At1g14410	263
bHLH	At2g20100	317	NAC	At3g44290	335	WHIRLY	At2g02740	268
bHLH	At2g38695	212	NAC	At3g49530	469	WRKY	At2g03340	513
bHLH	At4g38070	1513	NAC	At4g01540	473	YABBY	At1g08465	184
bHLH	At5g57150	226	NAC	At4g01550	457	YABBY	At1g23420	231
bZIP	At1g42990	295	NAC	At4g29230	498	ZF	At1g32540	328
bZIP	At2g40950	721	NAC	At4g35580	512	ZF	At2g26135	384
bZIP	At3g10800	675	NAC	At5g04400	395	ZF	At2g29660	373
bZIP	At3g56660	620	NAC	At5g04410	567	ZF	At2g38185	441
bZIP	At4g37730	305	NAC	At5g22290	340	ZF	At3g10470	398
bZIP	At5g28770	250	NAC	At5g24590	451	ZF	At3g20880	412
CAMTA	At4g16150	906	PHD	At2g37520	854	ZF	At3g47550	249
DUF231	At3g55990	487	PHD	At3g11200	233	ZF	At4g16141	226
GC-rich	At5g09210	603	PHD	At3g53680	839	ZF	At5g05660	880
Heat shock	At4g11660	377	PHD	At4g14920	1055	ZF	At5g40710	272
Homeodomain	At4g02560	953	PHD	At5g12400	1595	ZF	At5g63280	271
JUMONJI	At1g30810	787						

The *Arabidopsis* genome encodes more than 85 putative MTFs. The protein structures of the MTFs are displayed in Fig. 1.

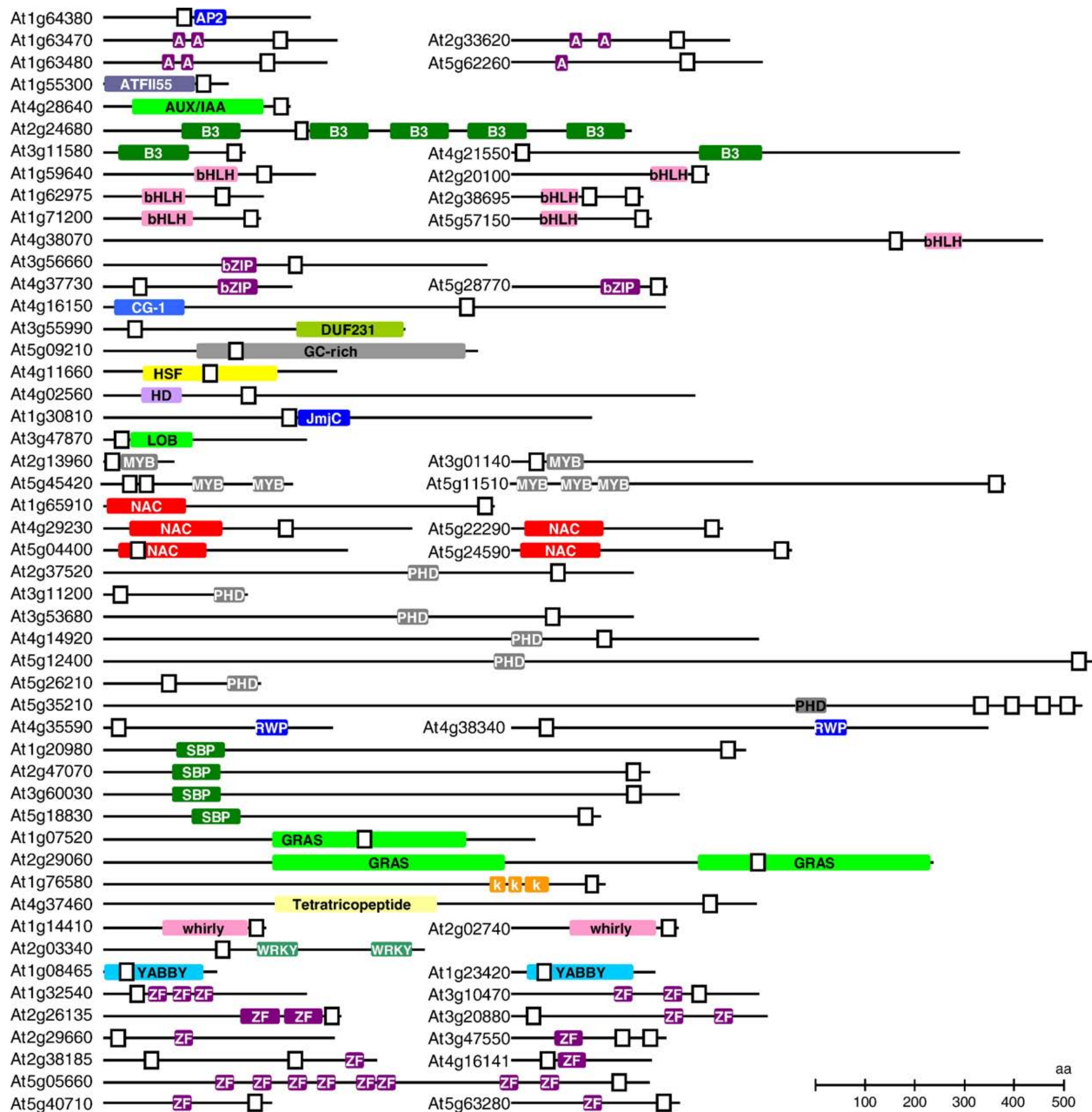

Fig. 1. Protein structures of putative *Arabidopsis* MTFs. The ARAMEMNON and TAIR databases were used to identify MTFs. Those having hydrophobicity values of higher than 0.85 were chosen. Protein domain structures were obtained from the Pfam protein family database (http://pfam.sanger.ac.uk/). The previously reported 13 NAC MTF (NTL) and 3 bZIP MTF members were not included in the list. AP2, APETALA2; A, AT HOOK; TAFII 55, TBP associated factor II 55; bHLH, basic helix-loop-helix; bZIP, basic leucine zipper motif; HSF, heat shock protein; HD, homeodomain; JmjC, JUMONJI C domain; LOB, lateral organ boundaries; NAC, NAM/ATAF1/2/CUC2; PHD, plant homeodomain; RWP, RWP-RK; SBP, squamosa promoter-binding protein; GRAS, GAI/RGA/SCR; k, ankyrin; ZF, zinc finger. Open boxes indicate transmembrane motif (TM). Numbers indicate residue positions.

the TM motif, raising a possibility that the transcription factor activity would also be regulated at the protein level through protein–protein interactions between the full-size and the alternatively spliced forms (see Discussion).

The rice genome also contains considerable number of putative MTFs. More than 45 MTFs were predicted in most of major transcription factor families (Table 2), as in the *Arabidopsis* genome. The prediction indicates that approximately 2% (45/2380) of total transcription factors (http://drtf.cbi.pku.edu.cn/) in rice are membrane-associated [25]. Protein sizes and locations of the TM motifs in the rice MTFs were also divergent similar to those observed in the *Arabidopsis* MTFs (Fig. S3). The protein sizes of the rice MTFs ranged from 209 to 1286 residues. Positions of the TM motifs were similar in the NAC and SBP MTFs of *Arabidopsis* and rice. Similarly, relative frequencies of MTF occurrence were higher in the PHD and NAC families, which contained six and five MTFs, respectively. Notably, a portion of the rice *MTF* genes is also alternatively spliced (Fig. S4), as observed with the *Arabidopsis MTF*s. Together, our observations indicate that not a few members of the *Arabidopsis* and rice transcription factors are membrane-associated. It is also envisioned that membrane regulation of transcriptional control of gene expression constitutes an additional way of regulating genome function in higher plants.

MTF genes are expressed in diverse plant tissues

Previous reports on plant MTFs have shown that they regulate diverse aspects of plant stress responses [5], including cell cycle control under stress conditions [15], ER stress responses [12,13,27], and flowering initiation and seed germination under high salinity [16]. In addition, they are expressed in specific plant organs, where they play major roles in mediating responses of such organs to external stress signals.

Table 2
Membrane-bound transcription factors in rice.

TF family	Locus (TIGR)	Size (aa)	TF family	Locus (TIGR)	Size (aa)
AP2	Os05g03040	361	NAC	Os08g06140	729
AP2	Os06g06970	253	NAC	Os08g44820	656
AP2	Os08g42550	541	NAC	Os09g32040	702
AP2	Os10g41330	273	PHD	Os01g11952	991
AUX/IAA	Os05g08570	251	PHD	Os01g46700	1015
AUX/IAA	Os05g14180	235	PHD	Os01g66420	272
B3	Os03g42370	1029	PHD	Os03g60390	247
B3	Os03g42410	306	PHD	Os05g07040	258
bHLH	Os01g39330	454	PHD	Os07g12910	244
bZIP	Os05g34050	646	SBP	Os01g18850	862
bZIP	Os06g41770	372	SBP	Os03g61760	969
bZIP	Os07g44950	568	SBP	Os05g33810	842
HAP2	Os03g07880	209	SBP	Os08g40260	1140
GRAS	Os12g06540	462	WRKY	Os01g08710	424
JUMONJI	Os01g67970	1286	WRKY	Os04g39570	998
JUMONJI	Os05g10770	1238	WRKY	Os05g03900	246
LIM	Os03g16090	501	WRKY	Os08g17400	550
LUG	Os01g08190	757	ZF-HD	Os08g34010	332
MADS	Os01g67890	483	ZF	Os01g06550	909
MADS	Os06g45650	221	ZF	Os01g15460	464
MYB	Os02g45080	253	ZF	Os06g40960	445
NAC	Os01g15640	489	ZF	Os07g38090	657
NAC	Os02g57650	632			

The rice genome encodes at least 45 putative MTFs. The protein structures of the rice MTFs are displayed in Fig. S3.

To obtain insights into physiological roles of plant MTFs, we examined expression patterns of plant *MTF* genes in different plant organs by digital Northern blot analyses using the GENEVESTIGATOR microarray database [24]. The results revealed that rice MTFs are classified into four major groups based on their expression patterns in different plant organs (Fig. S5).

Whereas some rice *MTF* genes are expressed in all plant organs, other genes are expressed in specific plant organs. Genes of group A are expressed mainly in seed embryo and endosperm, suggesting that these genes are involved in seed development and/or germination. Genes of group B are expressed in all plant organs with higher expression in floral organs. Those of group C were expressed primarily in floral organs. One of the group C genes, *Os04g39570* (WRKY), is highly expressed in stamen and anther, while a ZF transcription factor gene, *Os06g07880*, is predominantly expressed in pistil and stigma. In contrast, those of group D were expressed to high levels in vegetative organs, such as shoot and root.

Arabidopsis MTF genes are also expressed in various plant organs, individual genes with apparent predominance in specific organs (Fig. S6), like rice *MTF* genes. However, overall expression patterns of *Arabidopsis MTF* genes are more complicated compared to those of rice *MTF* genes. This may be because more microarray data have been accumulated and thus expression patterns of individual genes have been studied more extensively in *Arabidopsis*. Together, our data support that plant MTFs play diverse role in different plant organs in response to various stress conditions both in *Arabidopsis* and rice.

Rice genome encodes at least five NAC MTFs

Several members of the *Arabidopsis* NAC and bZIP family have been functionally studied in recent years. Furthermore, it has been estimated that among more than 100 NAC transcription factors in *Arabidopsis*, at least 18 NAC members are membrane-associated [3, Table 1]. Furthermore, it has been predicted that a few NAC members are likely to be membrane-associated in rice. We therefore decided to more systematically examine the rice NAC transcription factors to see whether the findings with *Arabidopsis* NACs are also applicable to rice NACs.

Our original prediction through analysis of protein domain structures and hydrophobicity profiling has shown that approximately six rice NAC members, designated OsNTLs, are integrated into the intracellular membranes [3]. More careful examination in this work showed that the gene structure of *OsNTL1* (*Os06g01230*) has been modified since the original prediction. The newly predicted protein did not contain any potential TM motifs. We therefore deleted the *OsNTL1* gene from the *OsNTL* gene members (Fig. 2A). The OsNTL proteins are relatively larger than *Arabidopsis* NTL proteins (Fig. 2A, Table 1) by consisting of 489–729 residues. The OsNTL3 protein is the smallest one among the OsNTL members and consists of 489 residues. Database searches revealed that it is equivalent to the OsNAC8 protein that has been previously studied [25]. The OsNTL2 has the largest size consisting of 729 residues.

While the sizes of OsNTL proteins and the gene structures are quite diverse (Figs. 2A and B), a common protein structure is shared by the OsNTL proteins. A DNA-binding NAC domain exists in the N-terminal region, and a strong α-helical TM domain having hydrophobicity values of higher than 0.85 exists near the C-terminal end in each OsNTL protein (Fig. 2C) [3]. The NAC domain consists of two major parts: one is a highly conserved DNA-binding domain and the other is a transcriptional activation domain [26]. The NAC domains of the OsNTL proteins also have similar protein structures, but the C-terminal sequences are highly diverse (data not shown), showing that overall architectures of the *Arabidopsis* and rice NTL proteins are quite similar to each other.

Phylogenetic analysis of the *Arabidopsis* NTL and OsNTL proteins by the BioEdit program (http://www.mbio.ncsu.edu/) showed that they are classified into four subgroups (Fig. 3). Interestingly, the four subgroups (TIP, NAC2, OsNAC8, and ANAC001) are clustered close to each other in the phylogenetic tree containing the whole NAC transcription factors [17,26]. It is therefore envisaged that the *Arabidopsis* and rice NTLs share some common roles, for example, regulatory roles in plants responses to environmental stress conditions. This notion is also supported by recent findings on the roles of NAC and bZIP MTFs in *Arabidopsis* [3,5,12–16].

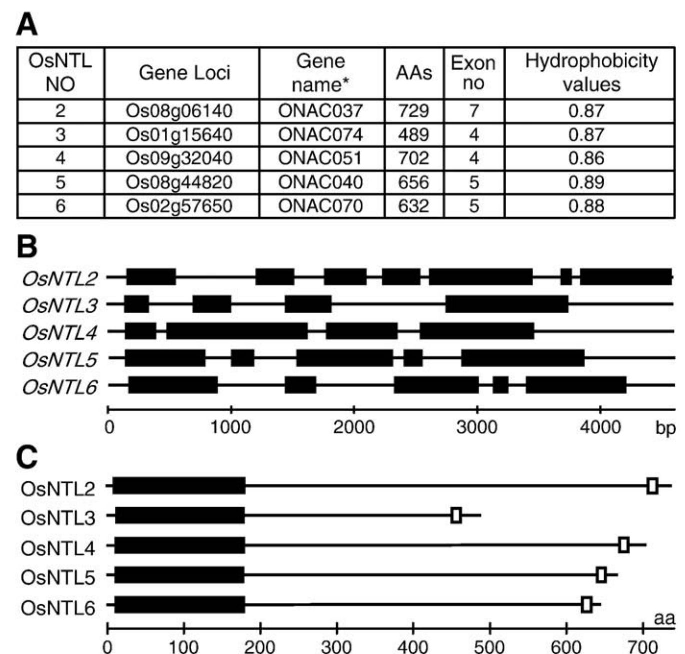

Fig. 2. Rice genome contains at least five NAC MTFs (OsNTLs). (A) *OsNTL* genes. The TMs of the OsNTL proteins have hydrophobicity values of higher than 0.85. *Gene names were annotated according to Ooka et al. [26]. Note that the OsNTL1 protein has been removed from the database after the original prediction [3]. (B) Exon–intron structures of *OsNTL* genes. The *OsNTL* genes consist of four to seven exons (black boxes). (C) Protein domain structures of OsNTL proteins. The NAC domains (black boxes) are located in their N-terminal regions, and the a-helical TMs (open boxes) are located in their C-terminal regions. Numbers indicate residue positions.

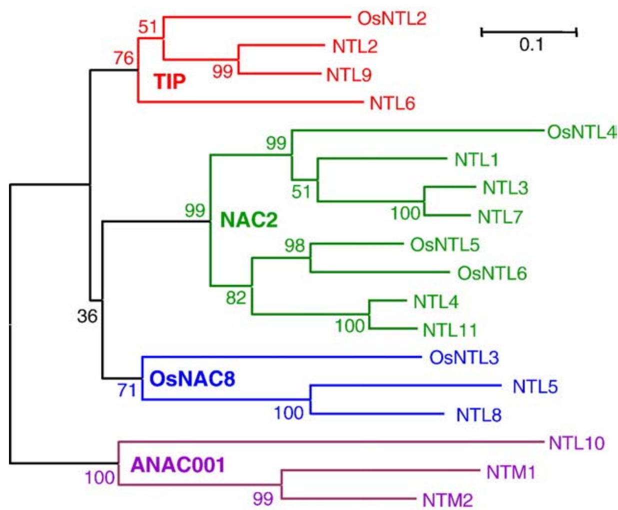

Fig. 3. Phylogenetic analysis of *Arabidopsis* NTL and OsNTL proteins. The amino acid sequences of the NAC domains of NTLs and OsNTLs were analyzed using the BioEdit software (http://www.mbio.ncsu.edu/). Subfamily names were annotated according to Ooka et al. [26] with each family with different colors.

OsNTLs are membrane-bound transcription factors

The predicted OsNTL proteins possess TM motifs in their far C-terminal regions, suggesting that they are expressed as dormant, membrane-associated forms, from which transcriptionally active, nuclear forms would be released.

To examine this hypothesis, a MYC-coding sequence was fused in-frame to the 5′ end of the *OsNTL2* gene, and the gene fusion was transiently expressed in *Nicotiana benthamiana* leaves. The OsNTL2 proteins were detected by Western blot analysis using an anti-MYC antibody. The total cellular extracts contained four major bands, designated B1–B4 (Fig. 4A). The estimated size of the B2 band was similar to that of the MYC-OsNTL2 fusion protein (OsNTL2 80.2 kDa + 6X MYC 10.5 kDa = 90.7 kDa). While the B1 band, which was estimated to have a molecular mass of 110 kDa, is larger than B2, the B3 band (estimated molecular mass of 82 kDa) is smaller than B2. The B4 band had an estimated molecular mass of 45 kDa, which is close to that of other membrane-free NTL forms that have been fused with six copies of MYC [3,11,15]. These observations suggest that whereas the B2 band is a full-size, membrane-associated form, the B4 band would be a processed, nuclear form. Although the identities of the B1 and B3 bands are currently unclear and more works are required to determine the identities, it is possible that the B1 band would be a posttranslationally modified form, and the B3 band would be a processing intermediate.

To further examine the processing pattern of the OsNTL2 protein, we carried out cell fractional assays, as has been carried out with other *Arabidopsis* NTL proteins [11,15]. The B1–B3 bands were detected predominantly in microsomal fractions (Fig. 4B, lane M). In addition, they were extracted from microsomal fractions by SDS-containing buffer, indicating that they are membrane-associated (Fig. 4B, lane SD). The smallest B4 band was detected in total extracts but disappeared very quickly through the fractionation steps (Figs. 4A and B). The B4 band intensities were also variable, depending on protein extracts. It has been reported that processed, nuclear forms of plant MTFs are also unstable [4,7,15], suggesting that the B4 band is a soluble, nuclear form.

To confirm the membrane association of the OsNTL2 protein, a GFP-coding sequence was in-frame fused to the 5′ end of a full-size *OsNTL2* gene, and the fusion construct was transiently expressed in *Arabidopsis* protoplasts. The GFP signals were detected in the plasma membrane as well as in the nucleus (Fig. 4C). In contrast, a truncated OsNTL2 form containing residues 1–350 and lacking the TM were detected exclusively in the nucleus (Fig. 4C, right panel). These observations strongly support that OsNTL2 is expressed as a plasma membrane-associated form, from which a soluble, nuclear form is released and enters the nucleus.

NAC domain-containing proteins constitute one of the largest transcription factor families in *Arabidopsis* and rice. To examine whether the rice OsNTL proteins possess transcriptional activation activities, the *OsNTL2* and *OsNTL3* genes were fused in-frame to the gene sequence encoding the GAL4 DNA-binding domain (BD) in the yeast vector pGBKT7, and the fusion constructs were expressed in yeast cells (HIS^-, ADE^-, $LacZ^-$). Growth of yeast cells (Fig. 4D) and β-galactosidase activity assays (Fig. 4E) demonstrated that the OsNTL2 and OsNTL3 proteins possess transcriptional activation activities. The relatively lower activities of the OsNTL2 and OsNTL3 proteins than that of the positive control (P) would be because full-size *OsNTL* genes, rather than those encoding truncated forms devoid of the TM domains, were used for yeast transformation, as has been observed with the NTM1 protein [15]. Although it was not examined, other rice OsNTL proteins are also expected to possess transcriptional activation activities.

The OsNTL genes exhibit diverse tissue-specific expression patterns

To obtain insights into the functional roles of the *OsNTL* genes, expression patterns of individual *OsNTL* genes were investigated by quantitative real-time RT-PCR (qRT-PCR) in different developmental stages and plant tissues. The overall expression patterns were quite similar for individual *OsNTL* genes. The *OsNTL2* gene was expressed to a similar level in all plant tissues except for seedling roots, where its expression level was relatively lower (Fig. 5A). Other *OsNTL* genes were highly expressed in the leaf tissues. In contrast, their transcript levels were relatively lower in the seedling leaves like the *OsNTL2* gene. Notably, transcript levels of all the *OsNTL* genes were relatively lower in the seedling roots. These results suggest that the *OsNTL* genes play a major role in later stages of leaf development or in responses of adult rice plants to environmental stresses as in Arabidopsis [3,5,12–16].

Analysis of temporal expression patterns revealed that the *OsNTL* genes exhibit similar expression patterns: they are expressed to a relatively lower level in the shoot base and young leaves. In contrast, their transcript levels were relatively higher in the late stages of leaf growth (Fig. 5B). These data suggest that the *OsNTL* genes function primarily in the late stages of leaf development.

OsNTL genes are induced by diverse abiotic stress conditions

The *Arabidopsis NTL* genes play roles in diverse aspects of plant responses to stressful conditions, and accordingly they are induced by a variety of biotic and abiotic stress conditions [3,17]. It was therefore envisioned that the rice *NTL* genes may also be influenced by environmental signals.

Wild-type rice plants grown for 1 week on Murashige and Skoog (MS)-agar plates were exposed to various abiotic stress conditions for appropriate time periods before harvesting plant materials for extraction of total RNA samples. Gene expression analyses by qRT-PCR showed that the *OsNTL2*, *OsNTL3*, *OsNTL4*, and *OsNTL5* genes were induced by high salinity and mannitol (Fig. 6). The *OsNTL6* gene was also induced under similar growth conditions but to a lesser degree. The *OsNTL3* and *OsNTL4* genes were also induced to a discernible degree by cold (4 °C) and heat (37 °C). These observations suggest that the *OsNTL* genes may play a role in a broad spectrum of plant stress responses, particularly in response to high salinity or drought. Overall, the fold changes were statistically significant, although not drastic. This may be because proteolytic processing is a major scheme regulating the OsNTL activities.

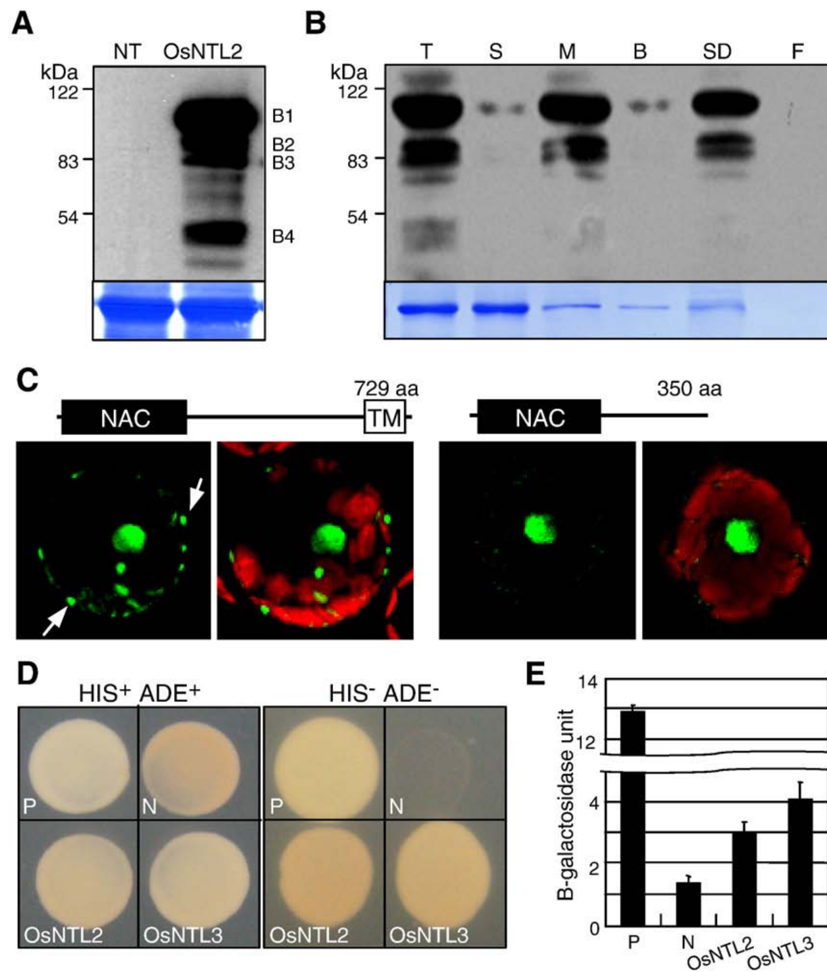

Fig. 4. The OsNTL proteins are membrane-associated transcription factors. (A) OsNTL2 processing. The *MYC-OsNTL2* fusion was transiently expressed in *N. benthamiana* leaves. The OsNTL2 proteins were detected by Western blot analyses using an anti-MYC antibody. Note that four major OsNTL2 forms, B1–B4, were detected. A part of Coomassie blue-stained membrane is displayed as loading control at the bottom. NT: total cellular extract of non-transformed leaves; OsNTL2, total cellular extract of *MYC-OsNTL2*-expressing leaves. kDa, kilodalton. (B) Cell fractionation assays. Cellular fractions were extracted from the infiltrated *N. benthamiana* leaves transiently expressing OsNTL2, as described by Kim et al. [15]. The OsNTL2 proteins were detected by Western blot analyses using an anti-MYC antibody. A part of Coomassie blue-stained membrane is displayed as loading control at the bottom. T, total extract; S, soluble fraction; M, membrane fraction; B, buffer-extracted fraction; SD, SDS-extracted fraction; F, final membrane fraction. (C) Subcellular localization of GFP-OsNTL2 and GFP-OsNTL2ΔC. The fusion constructs were transiently expressed in *Arabidopsis* protoplasts. Photographs were taken 16 h after transient transformation. Arrows indicate the location of membrane-associated OsNTL2. TM, transmembrane motif. (D) Transcriptional activation activity assays of OsNTL2 and OsNTL3. The transformed cells expressing OsNTL2 and OsNTL3 grown on the selective media without histidine and adenine (HIS^-ADE^-) clearly showed *LacZ* activation. (E) β-Galactosidase activity assays. Five independent measurements were averaged ($P<0.01$). Bars indicate standard error of the mean. Statistical significance was determined by a Student's *t*-test ($P<0.01$). P, positive control (full-size GAL4); N, negative control (DNA binding domain alone).

Dimethyl sulfoxide (DMSO) reduces membrane fluidity and is frequently used as a pharmacological reagent that mimics cold effects on plant cells. We examined the effects of DMSO on the expression of the *OsNTL* genes (Fig. 6). None of the *OsNTL* genes were affected by DMSO. Submergence and desiccation of the rice plants had no effect on the expression of the *OsNTL* genes as well.

Discussion

Numerous transcription factors are membrane-associated in Arabidopsis and rice

In this work, we carried out computer-assisted genome-scale screening and molecular and biochemical assays to identify transcription factors that are associated with the intracellular membranes in *Arabidopsis* and rice. Analyses of protein structures and hydrophobicity profiling estimated that there are at least 85 MTFs and 45 MTFs, constituting approximately 5% and 2% of total transcription factors, in the *Arabidopsis* and rice genomes, respectively. The number of the MTFs would be underestimated in this work. While we used a cut-off of 0.85 for hydrophobicity values, transcription factors that possess hydrophobicity values of lower than 0.85 would also be membrane-associated. It is expected that more MTFs would be identified as individual transcription factors are characterized in the future.

The MTF proteins are stored in their dormant forms in association with the intracellular membranes, such as nuclear membranes, plasma membranes, or ER membranes. When plants are exposed to abrupt environmental changes, they are released from the membranes through proteolytic cleavage events and enter the nucleus, where they regulate expression of genes involved in perception of stress signals, stress signaling, and stress responses of plant cells.

Membrane release of the AtbZIP60 and AtbZIP28 proteins is activated by tunicamycin, which induces ER stress [13,27]. The AtbZIP17 and NTL8 proteins are activated by high salt [14,16]. While several plant MTFs have been functionally characterized, more works are required to elucidate how the cleavage events are regulated and how the proteases are induced to cleave the MTFs. These questions would be answered after more proteases responsible for MTF activation are discovered in plants.

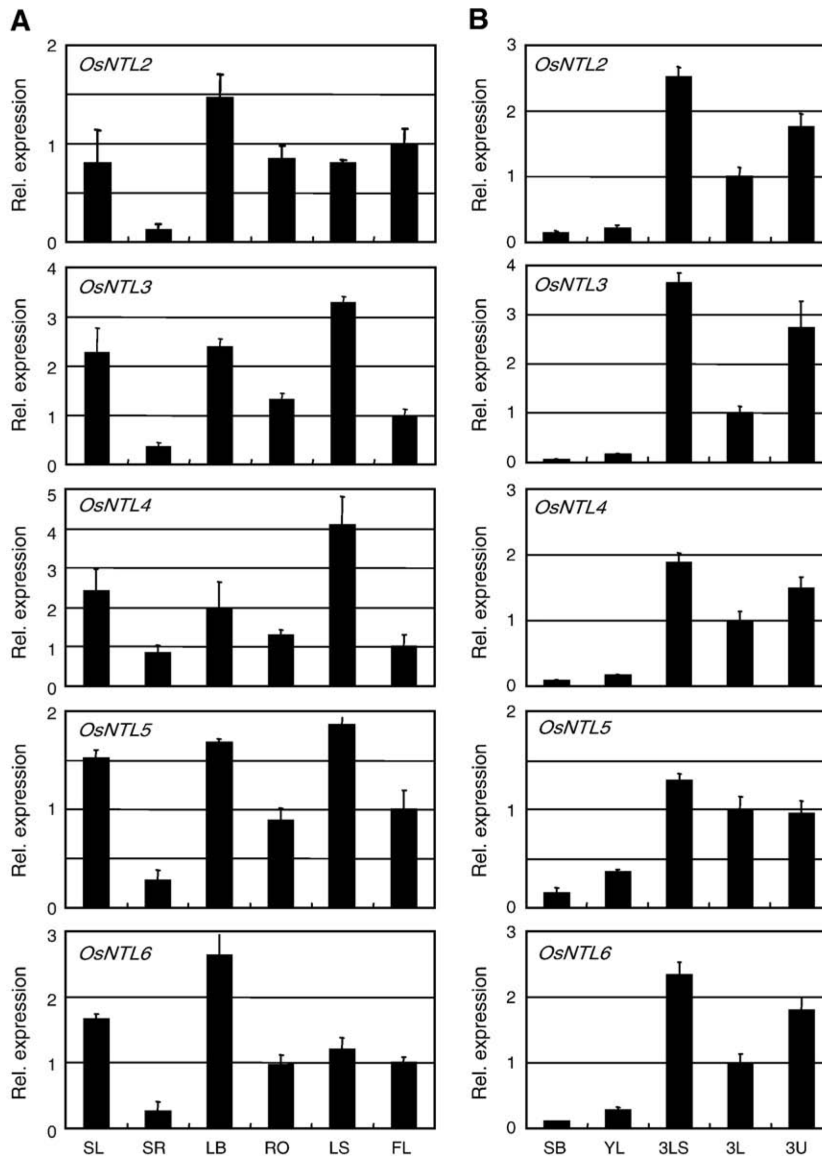

Fig. 5. Expression of *OsNTL* genes is spatially and temporally regulated. Rice plants were grown in a climate chamber set at a relative humidity of 70% under short days. The daily cycle was 10-h light at 30 °C and 14-h dark at 26 °C. Total RNA samples were extracted from appropriate plant materials. Transcript levels were determined by quantitative real-time RT-PCR (qRT-PCR). Biological triplicates were averaged. Bars indicate standard error of the mean. (A) Tissue-specific expression patterns. SL, seedling leaf; SR, seedling root; LB, leaf blade; RO, root; LS, leaf sheath; FL, flower. (B) Growth stage-dependent expression patterns. SB, shoot base; YL, young leaf; 3LS, leaf sheath of the third leaf; 3L, lower half of leaf blade of the third leaf; 3U, upper half of leaf blade of the third leaf.

While transgenic expression of foreign genes is widely used to improve plant resistance to biotic or abiotic stresses, overexpression of stress-resistant genes frequently reduces plant growth and seed production [28–30]. It has been reported that transgenic *Arabidopsis* plants overexpressing full-size *NTL* genes do not exhibit any discernible phenotypes [3,15,31], strongly supporting that the primary regulatory scheme for the MTF activities is the membrane-releasing step. It is expected that while transgenic rice expressing full-size *MTF* genes would phenotypically indistinguishable from wild-type plants under normal growth conditions, the transgenic rice plants would show an enhanced resistance to stresses, or they would respond more quickly to sudden environmental changes.

MTF activation mechanisms are conserved in animals and plants

Transcriptional activity assays in yeast cells demonstrated that the OsNTL2 and OsNTL3 proteins have transcriptional activation activities. They are expected to be membrane-associated in yeast cells. A question was how they regulate the expression of the reporter gene. It may be because the OsNTL2 protein expressed in yeast cells enters the nucleus via the nuclear localization signal peptide present in the protein. However, it is unlikely, since a truncated OsNTL2 protein lacking the TM motif does not possess any detectable transcriptional activation activity in yeast cells (data not shown). Therefore, a plausible explanation is that at least some of the OsNTL2 proteins expressed in yeast cells are released from the membranes and enter the nucleus. We found that when we transiently expressed the MYC-OsNTL2 fusion proteins in the tobacco leaves, both the membrane-associated and membrane-free forms were detected (Fig. 4A).

In animals, MTFs are released from the membranes either by regulated intramembrane proteolysis or by regulated ubiquitin/proteasome-dependent processing. Recently, several research groups have reported that the *Arabidopsis* MTF proteins are processed by membrane-bound proteases via RIP [14,16]. The *Arabidopsis* genome

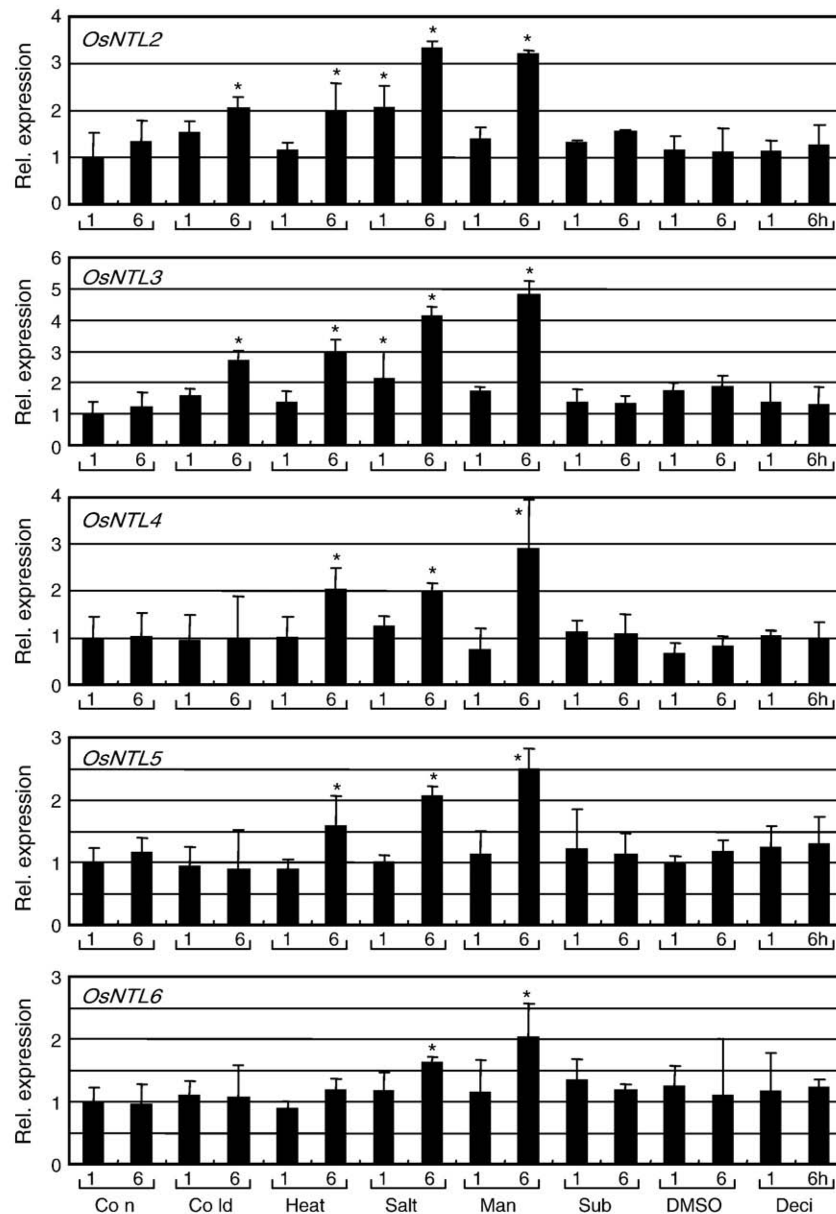

Fig. 6. The *OsNTL* genes are regulated by various abiotic stresses. Rice seedlings grown for 1 week on MS-agar plates were treated with different abiotic stresses for the indicated time periods. Total RNA samples were extracted from whole plants. Transcript levels were determined by qRT-PCR. Bars indicate standard error of the mean (*t*-test, *$P<0.05$). Con, control plants; Cold, cold treated plants at 4 °C; Heat, heat treated plants at 37 °C; Salt, salt (200 mM) treated plants; Man, mannitol (400 mM) treated plants; Sub, submerged plants; DMSO, 3% dimethyl sulfoxide treated plants; Desi, desiccated plants. h, hour.

encodes an animal S1P-like protease, which activates AtbZIP17 [14]. Although an *Arabidopsis* homologue of the S2P metalloprotease has been unknown, a metalloprotease activity induces NTL8 processing [16], suggesting that an unidentified metalloprotease would be responsible for processing of NTL8 and other NTL proteins in *Arabidopsis*.

OsNTL genes are induced by osmotic stress

Arabidopsis NAC transcription factors regulate a wide array of plant developmental processes, including stem cell formation, lateral root formation, and vascular development [32–35]. Interestingly, many of the NAC transcription factors are closely related with plant stress responses in *Arabidopsis* and rice [17,36,37]. Rice genome encodes five NAC domain-containing MTFs. Protein structures of the OsNTL proteins are similar to those observed in the *Arabidopsis* NTL proteins [3, this work]. The *OsNTL* genes are influenced by abiotic stress conditions, like the *Arabidopsis NTL* genes [3,31]. Collectively, these observations strongly support the notion that controlled activation of NAC MTFs, perhaps other membrane-associated transcription factors too, are conserved in dicotyledons and monocotyledons.

The *OsNTL* genes are induced by high salinity and mannitol, suggesting that they play a role in plant responses to osmotic stress. Salt accumulation in the rice leaves result in elevated synthesis of reactive oxygen species, defective photosynthesis, and abnormal chloroplast development [38,39], which eventually reduce crop productivity [40]. Induction of the *OsNTL* genes and enhanced activation of the OsNTL proteins would contribute to plant adaptation to osmotic stresses. Molecular genetic and transgenic approaches will be required to examine whether osmotic stresses promote proteolytic activation of the membrane-associated OsNTL proteins, as has been done in *Arabidopsis* [3,5,12–16].

Materials and methods

Bioinformatics softwares

The ARAMEMNON membrane protein database (http://aramemnon.botanik.uni-koeln.de/) was used to screen transcription factors that are predicted to be associated with the intracellular membranes in *Arabidopsis* and rice [21]. Amino acid sequences and gene annotations of the *Arabidopsis* MTFs were obtained from the *Arabidopsis* Information Resource (TAIR) database (http://www.arabidopsis.org/). Gene structures, amino acid sequences, and gene annotations of the rice MTFs were obtained from the Rice Genome Annotation Project database, Michigan State University (http://rice.plantbiology.msu.edu/). The ConPred II web-based program was used to identify transmembrane motifs (TMs) [23]. Phylogenetic trees were generated using the BioEdit analysis program (http://www.mbio.ncsu.edu/).

Screening of *Arabidopsis* and rice MTFs were carried out according to the following procedure. First-step selection of putative MTFs was based on the ARAMEMNON database that includes predicted membrane-associated proteins in *Arabidopsis* and rice [21]. Transcription factors having TM motifs with hydrophobicity values higher than 0.5 were collected from the primary screening. Next screening was performed via the method of Kyte–Doolittle [41]. Amino acid sequences of the selected *Arabidopsis* TFs were obtained from the TAIR database, and those of the rice TFs were acquired from the Database of Rice Transcription Factor (DRTF) [22]. The hydrophobic scales of the sequences were obtained using the ConPred II program [23]. We further selected putative MTFs, whose hydrophobic scales are higher than 1.5. The final screening step utilized the Gene Ontology database in the TAIR web site, from which we were able to collect total membrane proteins and total transcription factors. We selected those included in both the protein groups using a simplified visual basic program, which is operated through the EXCEL program as described in more detail in Fig. S1.

Analysis of transcript levels

Quantitative real-time RT-PCR (qRT-PCR) was employed to measure transcript levels. Total RNA samples were extracted from appropriate plant materials using the RNeasy Plant Total RNA Isolation Kit (Qiagen, CA). The first-strand cDNA was synthesized from 1 μg of total RNA using the Superscript II reverse transcriptase (Invitrogen, CA).

qRT-PCR was carried out in 96-well blocks with an Applied Biosystems 7500 Real-Time PCR System using the SYBR Green I master mix in a volume of 20 μl. The two-step thermal cycling profile consisted of 15 s at 94 °C and 1 min at 68 °C. All the reactions were performed in biological triplicates using three RNA samples extracted from independent plant materials. An *UBQ5* gene (At3G62250) was included in the assays as internal control for normalizing the variations in cDNA amounts used. The comparative $\Delta\Delta C_T$ method was used to evaluate the relative quantities of each amplified product in the samples. The threshold cycle (C_T) was automatically determined for each reaction by the system set with default parameters. The specificity of the qRT-PCR reactions was determined by melt curve analysis of the amplified products using the standard method installed in the System.

OsNTL processing

For analysis of OsNTL processing in *N. benthamiana* leaf cells, the *OsNTL2* gene was subcloned into the pBA002-6XMYC vector that contains six copies of MYC-coding sequence [15]. *Agrobacterium tumefaciens* cells harboring the expression construct were injected directly into the leaves using syringe without needle. After incubation for 24 h at room temperature, the leaves were ground in liquid nitrogen, and the total cellular extracts were suspended in 1× SDS-PAGE sample loading buffer. The OsNTL2 proteins were immunologically detected using an anti-MYC antibody (Santa Cruz Biotech., CA). Cell fractionation assays were carried out as previously described [15].

Subcellular localization of OsNTL proteins

The green fluorescence protein (GFP)-coding sequence was in-frame fused to the 5′ end of *OsNTL2* and *OsNTL2ΔC* genes, and the gene fusion constructs were transiently expressed in *Arabidopsis* protoplasts [42]. After incubation for 16 h at room temperature in the dark, the cells were observed using the Multi-photon Confocal Laser Scanning microscope (LSM510 NLO, Carl Zeiss, Germany).

Transcriptional activation activity assays

Transcriptional activation activity assays were performed essentially as described previously [16]. The pGBKT7 vector and the yeast strain AH109 were used (Clontech, CA). The *OsNTL2* and *OsNTL3* gene sequences were fused to the GAL4 DNA binding domain-coding sequence of pGBKT7, and the vector constructs were transformed into the AH109 cells. Five measurements were averaged and statistically treated using the Student's *t*-test.

Plant materials and treatments with abiotic stresses

The rice (*Oryza sativa*) var. japonica ‘Dongjin’ plants were grown in a climate chamber set at a relative humidity of 70% under short days with white light illumination (120 μmol photons/m^2 s, 400–700 nm) provided by fluorescent FLR40D/A tubes (Osram, Seoul, Korea). The daily cycle was 10-h light at 30 °C and 14-h dark at 26 °C. Plant materials were harvested throughout the growth stages until flowering.

For abiotic stress treatments, rice plants were grown in a climate chamber set at 28 °C with a relative humidity of 60% under long days (14-h light and 10-h dark). One-week-old seedlings grown in Murashige and Skoog (MS)-agar plates were used for all abiotic stress treatments. For cold and heat treatments, plants were placed at either 4 °C or 37 °C for the indicated time periods. For salt and mannitol treatments, plants were incubated either in the presence of 200 mM NaCl or 400 mM mannitol, respectively, for the indicated time periods. Plants were submerged in distilled water for submergence stress treatment. In order to reduce membrane fluidity, plants were treated with 3% dimethyl sulfoxide (DMSO) solution. For desiccation treatment, plants were dehydrated on dry 3 MM filters for the indicated time periods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2009.09.003.

References

[1] S. Ghosh, M.J. May, E.B. Kopp, NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses, Annu. Rev. Immunol. 16 (1998) 225–260.
[2] A. Kaffman, E.K. O'Shea, Regulation of nuclear localization, a key to a door, Annu. Rev. Cell Dev. Biol. 15 (1999) 291–339.
[3] S.Y. Kim, et al., Exploring membrane-associated NAC transcription factors in *Arabidopsis*: implications for membrane biology in genome regulation, Nucleic Acids Res. 35 (2007) 203–213.
[4] T. Hoppe, M. Rape, S. Jentsch, Membrane-bound transcription factors: regulated release by RIP or RUP, Curr. Opin. Cell Biol. 13 (2001) 344–348.
[5] P.J. Seo, S.G. Kim, C.M. Park, Membrane-bound transcription factors in plants, Trends Plant Sci. 13 (2008) 550–556.
[6] M.T. Bengoechea-Alonso, J. Ericsson, SREBP in signal transduction: cholesterol metabolism and beyond, Curr. Opin. Cell Biol. 19 (2007) 215–222.
[7] M.S. Brown, J. Ye, R.B. Rawson, J.L. Goldstein, Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans, Cell 100 (2000) 391–398.
[8] D. Eberle, B. Hegarty, P. Bossard, P. Ferre, F. Foufelle, SREBP transcription factors: master regulators of lipid homeostasis, Biochimie 86 (2004) 839–848.
[9] R.B. Rawson, Control of lipid metabolism by regulated intramembrane proteolysis of sterol regulatory element binding proteins (SREBPs), Biochem. Soc. Symp. 70 (2003) 221–231.
[10] J. Sakai, et al., Sterol-regulated release of SREBP-2 from cell membranes requires two sequential cleavages, one within a transmembrane segment, Cell 85 (1996) 1037–1046.
[11] J. Sakai, A. Nohturfft, J.L. Goldstein, M.S. Brown, Cleavage of sterol regulatory element-binding proteins (SREBPs) at site-1 requires interaction with SREBP cleavage-activating protein—evidence from in vivo competition studies, J. Biol. Chem. 273 (1998) 5785–5793.
[12] Y. Iwata, N. Koizumi, An *Arabidopsis* transcription factor, AtbZIP60, regulates the endoplasmic reticulum stress response in a manner unique to plants, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 5280–5285.
[13] Y. Iwata, N.V. Fedoroff, N. Koizumi, *Arabidopsis* bZIP60 is a proteolysis-activated transcription factor involved in the endoplasmic reticulum stress response, Plant Cell 20 (2008) 3107–3121.
[14] J.X. Liu, R. Srivastava, P. Che, S.H. Howell, Salt stress responses in *Arabidopsis* utilize a signal transduction pathway related to endoplasmic reticulum stress signaling, Plant J. 51 (2007) 897–909.
[15] Y.S. Kim, et al., A membrane-bound NAC transcription factor regulates cell division in *Arabidopsis*, Plant Cell 18 (2006) 3132–3144.
[16] S.G. Kim, A.K. Lee, H.K. Yoon, C.M. Park, A membrane-bound NAC transcription factor NTL8 regulates gibberellic acid-mediated salt signaling in *Arabidopsis* seed germination, Plant J. 55 (2008) 77–88.
[17] Y.J. Fang, J. You, K.B. Xie, W.B. Xie, L.Z. Xiong, Systematic sequence analysis and identification of tissue-specific or stress-responsive genes of NAC transcription factor family in rice, Mol. Genet. Genomics 280 (2008) 547–563.
[18] H.H. Hu, et al., Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 12987–12992.
[19] H.H. Hu, et al., Characterization of transcription factor gene *SNAC2* conferring cold and salt tolerance in rice, Plant Mol. Biol. 67 (2008) 169–181.
[20] K. Nakashima, et al., Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice, Plant J. 51 (2007) 617–630.
[21] R. Schwacke, et al., ARAMEMNON, a novel database for *Arabidopsis* integral membrane proteins, Plant Physiol. 131 (2003) 16–26.
[22] G. Gao, et al., DRTF: a database of rice transcription factors, Bioinformatics 22 (2006) 1286–1287.
[23] M. Arai, et al., ConPred II: a consensus prediction method for obtaining transmembrane topology models with high reliability, Nucleic Acids Res. 32 (2004) W390–W393.
[24] P. Zimmermann, M. Hirsch-Hoffmann, L. Hennig, W. Gruissem, GENEVESTIGATOR. *Arabidopsis* microarray database and analysis toolbox, Plant Physiol. 136 (2004) 2621–2632.
[25] K. Kikuchi, K. Harada, K. Yoshida, Y. Nagato, H. Hirano, Studies on structure and expression pattern of *OsNAC8* gene in rice, Breeding Res. 1 (1999) 118.
[26] H. Ooka, et al., Comprehensive analysis of NAC family genes in *Oryza sativa* and *Arabidopsis thaliana*, DNA Res. 10 (2003) 239–247.
[27] J.X. Liu, R. Srivastava, P. Che, S.H. Howell, An endoplasmic reticulum stress response in *Arabidopsis* is mediated by proteolytic processing and nuclear relocation of a membrane-associated transcription factor, bZIP28, Plant Cell 19 (2007) 4111–4119.
[28] Y.N. Chen, E. Slabaugh, F. Brandizzi, Membrane-tethered transcription factors in *Arabidopsis thaliana*: novel regulators in stress response and development, Curr. Opin. Plant Biol. 11 (2008) 695–701.
[29] J.J. Burdon, P.H. Thrall, The fitness costs to plants of resistance to pathogens, Genome Biol. 4 (2003) 227.1–227.3.
[30] J.E. Park, et al., GH3-mediated auxin homeostasis links growth regulation with stress adaptation response in *Arabidopsis*, J. Biol. Chem. 282 (2007) 10036–10046.
[31] H.K. Yoon, S.G. Kim, S.Y. Kim, C.M. Park, Regulation of leaf senescence by NTL9-mediated osmotic stress signaling in *Arabidopsis*, Mol. Cells 25 (2008) 438–445.
[32] J.L. Riechmann, et al., *Arabidopsis* transcription factors: genome-wide comparative analysis among eukaryotes, Science 290 (2000) 2105–2110.
[33] M. Aida, T. Ishida, H. Fukaki, H. Fujisawa, M. Tasaka, Genes involved in organ separation in *Arabidopsis*: an analysis of the cup-shaped cotyledon mutant, Plant Cell 9 (1997) 841–857.
[34] N. Mitsuda, et al., NAC transcription factors, NST1 and NST3, are key regulators of the formation of secondary walls in woody tissues of *Arabidopsis*, Plant Cell 19 (2007) 270–280.
[35] Q. Xie, G. Frugis, D. Colgan, N.H. Chua, *Arabidopsis* NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development, Genes Dev. 14 (2000) 3024–3036.
[36] M. Fujita, et al., A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway, Plant J. 39 (2004) 863–876.
[37] L. Tran, et al., Isolation and functional analysis of *Arabidopsis* stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promoter, Plant Cell 16 (2004) 2481–2498.
[38] M.L. Dionisio-Sese, S. Tobita, Antioxidant responses of rice seedlings to salinity stress, Plant Sci. 135 (1998) 1–9.
[39] K. Yamane, S. Rahman, M. Kawasaki, M. Taniguchi, H. Miyake, Pretreatment with antioxidants decreases the effects of salt stress on chloroplast ultrastructure in rice leaf segments (*Oryza sativa* L.), Plant Prod. Sci. 7 (2004) 292–300.
[40] W.X. Wang, B. Vinocur, A. Altman, Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance, Planta 218 (2003) 1–14.
[41] J. Kyte, R.F. Doolittle, A simple method for displaying the hydropathic character of a protein, J. Mol. Biol. 157 (1982) 105–132.
[42] J. Sheen, Signal transduction in maize and *Arabidopsis* mesophyll protoplasts, Plant Physiol. 127 (2001) 1466–1475.