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Supplementary Information for

Regulatory Cascade Involving Transcriptional and N-end Rule Pathways in Rice under Submergence

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References for SI reference citations

Other supplementary materials for this manuscript include the following:

Dataset S1

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Supplementary Text

SI Materials and Methods

Growth Conditions and Submergence Treatment for Arabidopsis

Arabidopsis seeds were sterilized with 0.5% (v/v) sodium hypochlorite for 15 min and washed with sterilized water. Seeds were sown on plates with 0.57% Phytagel (Sigma-Aldrich) in half-strength Murashige and Skoog (MS) medium (Duchefa Biochemie) containing 0.5% sucrose at pH 5.7 and kept at 4°C in the dark for three days to achieve uniform germination, then the plates were transferred to a growth chamber and grown at 22°C with a 16-h-light (81 $\mu mol \cdot m^{-2} \cdot s^{-1}$)/8-h-dark cycle for four days. To reach 14-d-old for submergence treatment, 7-d-old seedlings were transplanted onto fresh plates, and the plates were placed vertically to prevent roots from growing into medium. The transplanted seedlings were grown in the growth chamber until they were 14-d-old. For submergence treatment of 14-d-old Arabidopsis seedlings, plates with plants on the surface of the medium were placed into half-strength MS liquid medium that was bubbled with 3% oxygen balanced with nitrogen for the indicated times. The liquid MS medium was pretreated with 3% oxygen for 1 h before use.

RNA extraction and qRT-PCR

Total RNA from treated samples were isolated by using TRIzol (Invitrogen) following the manufacturer's instructions. DNase treatment, Reverse Transcription and qRT-PCR were conducted as previously described (61). Briefly, first-strand cDNAs were synthesized from 2 μg of extracted total RNA using M-MLV reverse transcriptase (Invitrogen). The synthesized cDNAs were further made a five-fold dilution. qRT-PCR was performed using 1 μL diluted cDNAs, 0.2 μM each primer, and SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7500 real time PCR machine (Applied Biosystems) using the default settings. Primer sequences can be found in Table S1. Tubulin (LOC_Os07g38730) was used as an internal control for normalization. Relative expression level is determined by ΔC_T of target gene normalized to the internal control.

RNA-seq and data analysis

Fourteen days old SUB1A-1 OE/ERF66 OE/ERF67 OE transgenic plants and non-transformed TNG67 (as a control) were submerged for 24 h, and the whole-shoot tissue was harvested before (T0) and after (T24) treatment to perform RNA extraction and RNA-seq. Total RNA from three independent biological samples were isolated by using TRIzol (Invitrogen) following the manufacturer's instructions. DNase treatment was conducted as previously described (61). RNA libraries were constructed by using Truseq Stranded mRNA library preparation kit (Illumina). All procedures followed the protocol provided by the manufacturer. The steps of library constructions included mRNA isolation, RNA fragmentation, cDNA synthesis, adaptor ligation and DNA enrichment, starting with 4 μg of total RNA. All sequencing data were generated using Illumina sequencing, following standard protocols, with the HiSeq2500 platform. Paired-end sequencing reads were trimmed and filtered using Trimmomatic (version 0.32) (62). The clean reads were mapped to the Os-Nipponbare-Reference-IRGSP-1.0 (IRGSP-1.0) annotated reference transcriptome from the Rice Annotation Project Database (RAP-DB, https://rapdb.dna.affrc.go.jp/) by Bowtie2 (63). Read counts were then analyzed for differentially expressed genes using DESeq2 (version 3.7) (64). Genes with a log2 fold-change above 1 (or below -1) and an adjusted P-value below 0.05 were defined as differentially expressed. Gene Ontology (GO) analyses of RNA-seq data were conducted using agriGO (http://bioinfo.cau.edu.cn/agriGO/index.php) (65) and choose $P<0.05$ as the cut-off for significant GO terms. Raw RNAseq data have been deposited in the National Center for Biotechnology Information (NCBI) BioProject database (Accession number: PRJNA512592; https://www.ncbi.nlm.nih.gov/sra/PRJNA512592).

Plasmid Construction

For *trans*-activation assays, effector and reporter vectors were generated following the procedures outlined in (66). We further incorporated the Gateway gene cloning system (Invitrogen) to generate the effector gateway vector, pUbiP-GW, and the reporter gateway vector, pGW-Luc. The pUbiP-GW contained *Ubiquitin* promoter, ccdB fragment and *Nos* terminator and pGW-Luc contained *CaMV35S* minimal promoter, *Adh1* intron, ccdB fragment and *luciferase* coding sequence, sequentially. The CDS of *SUB1A-1* was amplified by PCR using submerged FR13A cDNA as the template, and ligated into the pCR8/GW/TOPO (Invitrogen). By Gateway system (Invitrogen), the

SUB1A-1 DNA fragment was subcloned into pUbiP-GW. The 1.3-kb upstream promoter sequences of *ERF66* and *ERF67* were amplified by PCR from genomic DNA of FR13A and ligated into pCR8 vector followed by subcloning into pGW-Luc.

For protein stability assays, various constructs (Fig. S7B) were amplified using corresponding primers, and double digested with BamHI and HindIII, respectively. Luc were amplified from pGreenII 0800-Luc, and then double digested with HindIII and BamHI. Each gene construct together with Luc fragment, were co-ligated into pUbiP-NosT, which was digested with BamHI.

For recombinant protein expression, the pCR8-SUB1A-1 plasmids were used as template to amplified SUB1A-1 and SUB1A-1N DNA fragments by PCR, both of which were carrying EcoRV-TEV protease cleavage site and XhoI site at 5' and 3' end. After double digestion of EcoRV and XhoI, the SUB1A-1 and SUB1A-1N DNA fragments were ligated into pET32a vectors which were double digested with MscI and XhoI to produce thioredoxin (Trx) -TEV-SUB1A-1/SUB1A-1N-6xHis fusions driven by T7 promoter. All primers used for the cloning of cDNAs are listed in Table S2.

Trans-Activation Assay

The *trans*-activation assay was conducted as described (66) with minor modifications. TNG67 rice protoplast cells were co-transformed with the effector, reporter, and internal control plasmids at a mass ratio of 1:1:0.5, and incubated for 15 h. Protoplasts were collected by centrifugation for 15 s at 13,000 rpm, resuspended in 0.2 mL extraction buffer (100 mM potassium phosphate buffer, pH 7.8, 1 mM EDTA, 7 mM β-mercaptoethanol, 1% Triton X-100, and 10% glycerol), and vortexed for 15 seconds at high speed. The disrupted protoplasts were centrifuged at 13,000 rpm and 4°C for 5 min. The supernatant was collected and used for luciferase and GUS activity assays. For luciferase activity assay, 50 μL of cell extract was placed in a 96-well luminometry plate (Nunclon), then 90 μL of luciferase assay buffer (25 mM Tricine, pH 7.8, 15 mM potassium phosphate buffer, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 2 mM ATP, and 1 mM dithiothreitol) was added. The plate was placed into the VICTOR 3V Multilabel Plate Reader (Perkin Elmer), and 50 μL of 0.2 mM luciferin (Promega) was injected into the well to start the reaction. The photons emitted were integrated over a 10-s period and

expressed as a relative light units/10 s. For GUS activity assay, 25 μL of cell extract was placed in a 96-well fluorescence plate (Nunclon), then 25 μL 2× GUS assay buffer (100 mM sodium phosphate buffer, pH 7.0, 20 mM β-mercaptoethanol, 20 mM EDTA, 0.2% sodium lauryl sarcosin, 0.25% Triton X-100, and 2.5 mM 4MUG) was added and incubated at 37°C in the dark for 30 min. The reactions were stopped by adding 150 μL 0.2 M Na_2CO_3. Fluorescence (excitation at 355 nm and emission at 460 nm) was determined by the VICTOR 3V Multilabel Plate Reader.

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) was conducted as described (66) with minor modifications. Fluorescein amidite (FAM) labeled DNA probes were synthesized by PURIGO Biotechnology Co., Ltd.. DNA-protein binding reaction was carried out by incubation of 0.05 μM of FAM-labeled probe with 0.5 μM of recombinant SUB1A-1 protein in a total volume of 20 μl of solution containing 17 mM Hepes, pH 7.9, 60 mM KCl, 7.5 mM $MgCl_2$, 0.12 mM EDTA, 17% glycerol, and 1.2 mM dithiothreitol, 0.5 μg of poly(dI-dC)(Sigma). The assay mixture was incubated for 20 min at room temperature. Protein-DNA complexes were separated on a 6% native polyacrylamide gel after which the FAM signal was imaged using a Typhoon Scanner (GE Healthcare). All probes used herein are listed in Table S3.

Yeast Two-Hybrid Assays

The yeast two-hybrid experiment was conducted by using the GAL4-based yeast two-hybrid system (Matchmaker Gold Yeast Two-Hybrid System, Clontech). By Gateway system (Invitrogen), the fragment of SUB1A-1 N-terminus was subcloned into the pGADT7-DEST, and the fragments of SUB1A-1, ERF66 and ERF67 C-termini and were subcloned into the pGBKT7-DEST, respectively. pGADT7-AKIN10 (At3g01090.1) and pGBKT7-At5g20700 plasmids were used as positive bait and prey controls. pGADT7-T and pGBKT7-Lam plasmids were used as negative bait and prey controls, respectively. SD-WL (-Trp -Leu) plates were used to select for the presence of both bait and prey vectors, and SD-WLH (-Trp -Leu -His) plates were used to check the interactions between the bait and prey proteins.

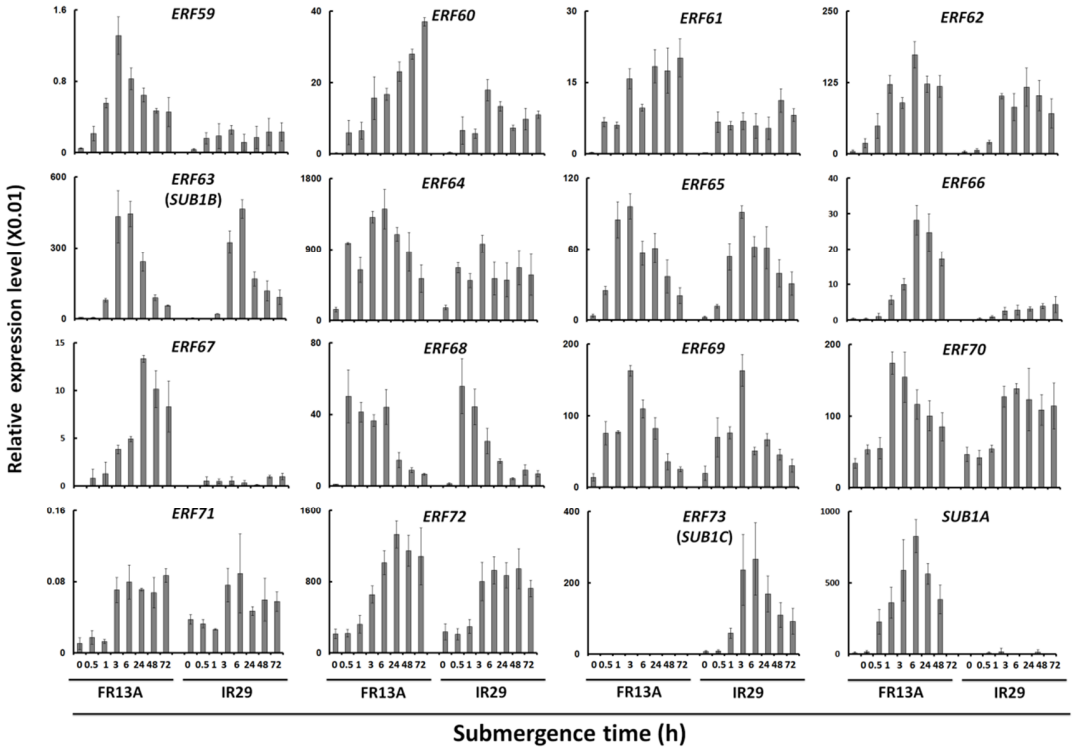


Fig. S1. Transcriptional profiles of *OsERFVIIs* under submergence in FR13A and IR29.

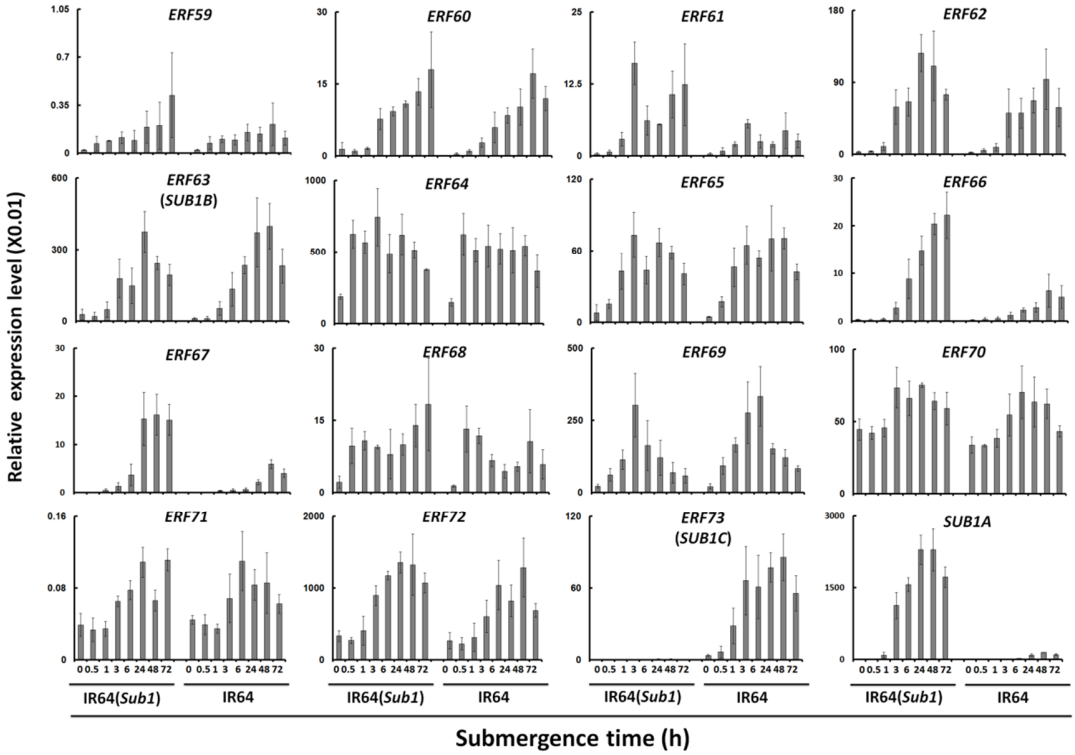


Fig. S2. Transcriptional profiles of *OsERFVIIs* under submergence in IR64(*Sub1*) and IR64.

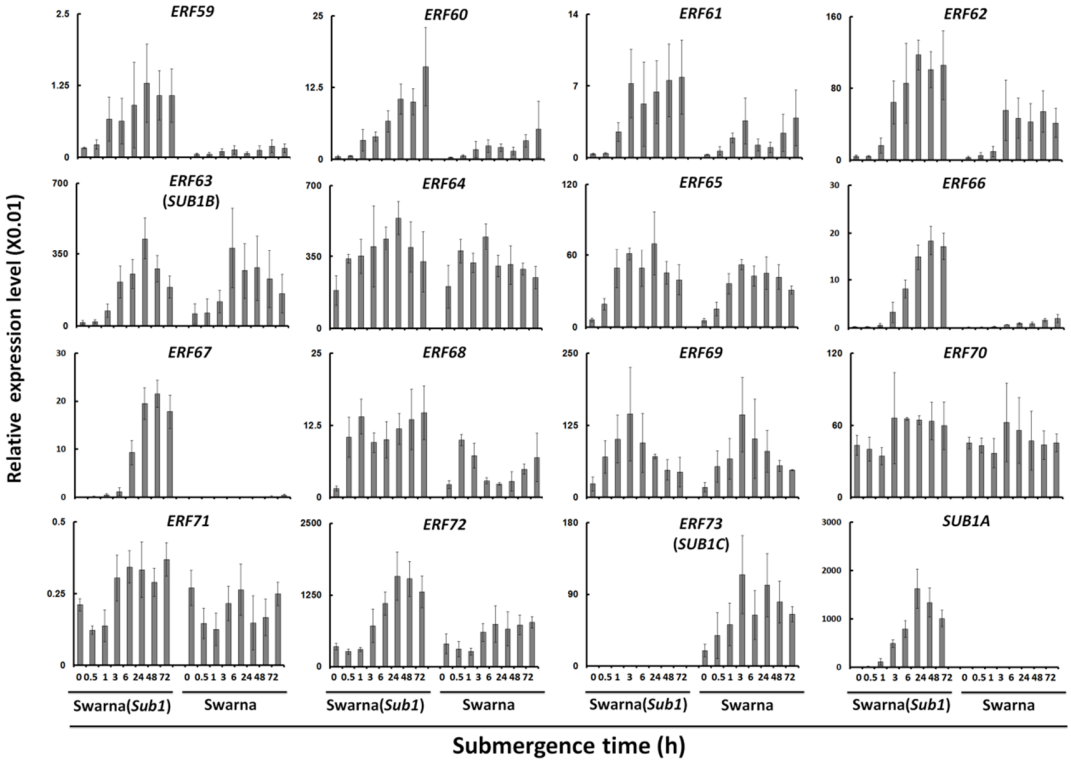


Fig. S3. Transcriptional profiles of *OsERFVIIs* under submergence in Swarna(*Sub1*) and Swarna.

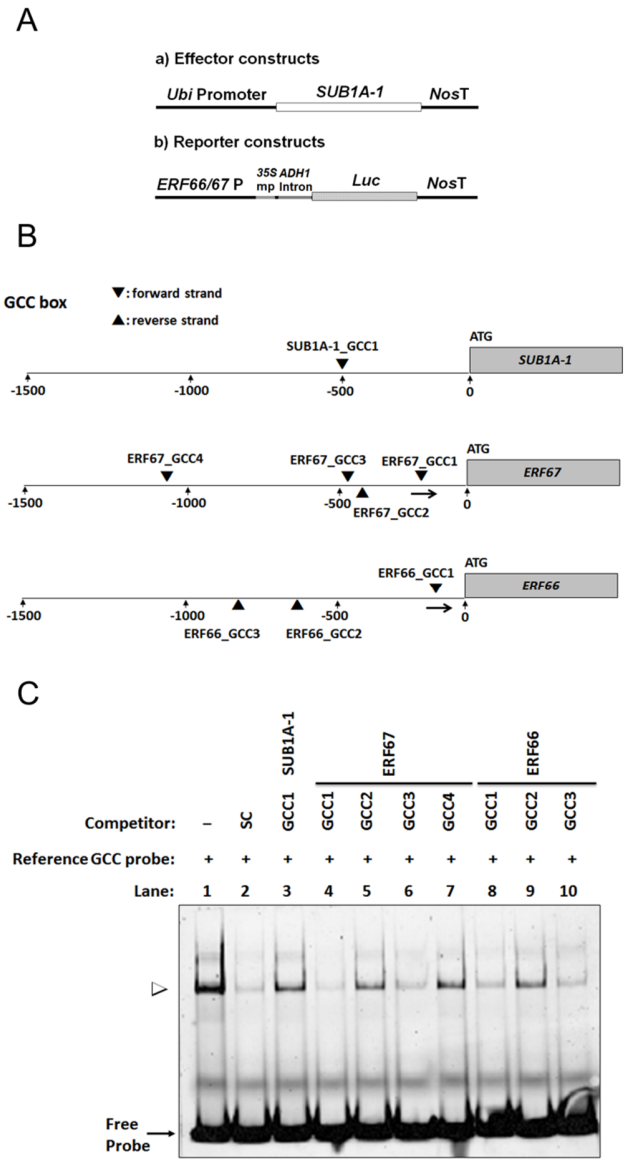
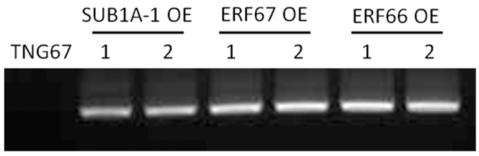


Fig. S4. Schematic diagrams of constructs used in transactivation assay and GCC boxes in promoter of *SUB1A-1*, *ERF67* and *ERF66*. (A) Effector and reporter constructs are illustrated. (B) The diagram shows the positions of GCC boxes in the promoters of *SUB1A-1*, *ERF67* and *ERF66*. The flanking sequence of these GCC boxes can be found in Table S3. The arrows indicate the positions of the primer set of ChIP-qPCR. (C) Competition EMSA assays of recombinant SUB1A-1 and FAM-labeled reference GCC DNA (60), using unlabeled DNA for competitive binding. The unlabeled DNA used for competitive binding was in 100-fold excess (W/W) of the FAM-labeled reference GCC DNA. Lane 1, without competitor. Lane 2, unlabeled reference GCC DNA (SC: specific competitor). Lane 3 to 10, unlabeled DNA (upper panel B and Table S3). The triangle indicates the position of the DNA-protein complexes. The arrow indicates the free probes.

A



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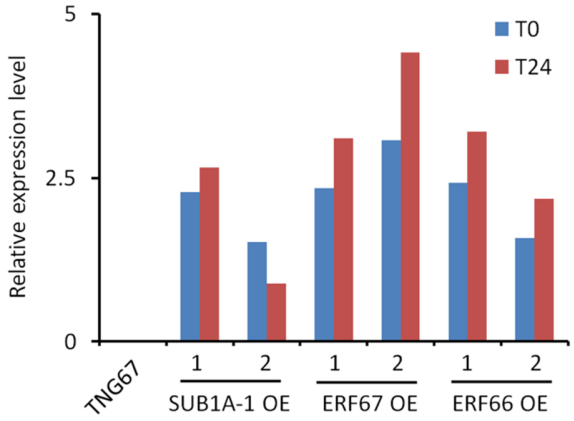

C

Line		TNG67	SUB1A-1 OE		ERF67 OE		ERF66 OE	
			1	2	1	2	1	2
Repeat 1	Number of plant treated	26	13	19	17	30	15	19
	Number of plant survived	1	8	12	6	24	6	13
	Viability (%)	3.8	61.5	63.2	35.3	80.0	40.0	68.4
Repeat 2	Number of plant treated	29	24	24	23	33	22	20
	Number of plant survived	2	19	19	17	16	13	16
	Viability (%)	6.9	79.2	79.2	73.9	48.5	59.1	80.0
Average viability (%)		5.4	70.4	71.2	54.6	64.2	49.5	74.2
SD		1.5	8.8	8	19.3	15.8	9.5	5.8

Fig. S5. The profiles of SUB1A-1, ERF67, ERF66 overexpression transgenic rice lines. These lines were generated by individually transforming the *UbiP::GST-SUB1A-1/ERF67/ERF66* in pCAMBIA1301 vector into TNG67 rice. (A) Genomic PCR of hygromycin selection marker of 14 days old SUB1A-1 OE/ERF67 OE/ERF66 OE transgenic plants. (B) Transcript levels of transgenic GST tagged SUB1A-1/ERF67/ERF66 were detected by qRT-PCR using GST specific primers (Table S1). Fourteen days old SUB1A-1 OE/ERF67 OE/ERF66 OE transgenic plants and non-transformed TNG67 were submerged for 24 h, and the whole-shoot tissue was harvested before (T0) and after (T24) treatment to perform qRT-PCR. Relative expression level is determined by delta CT of GST normalized by Tubulin mRNA level as the internal control. (C) The viability test of SUB1A-1, ERF67, ERF66 overexpression transgenic rice lines. Two independent replicates were performed. The table shows the number of plants treated, number of plant survived, and viability of each transgenic line in each independent replicate. The average viability and SD of these two replicates shown herein is related to Fig. 3B.

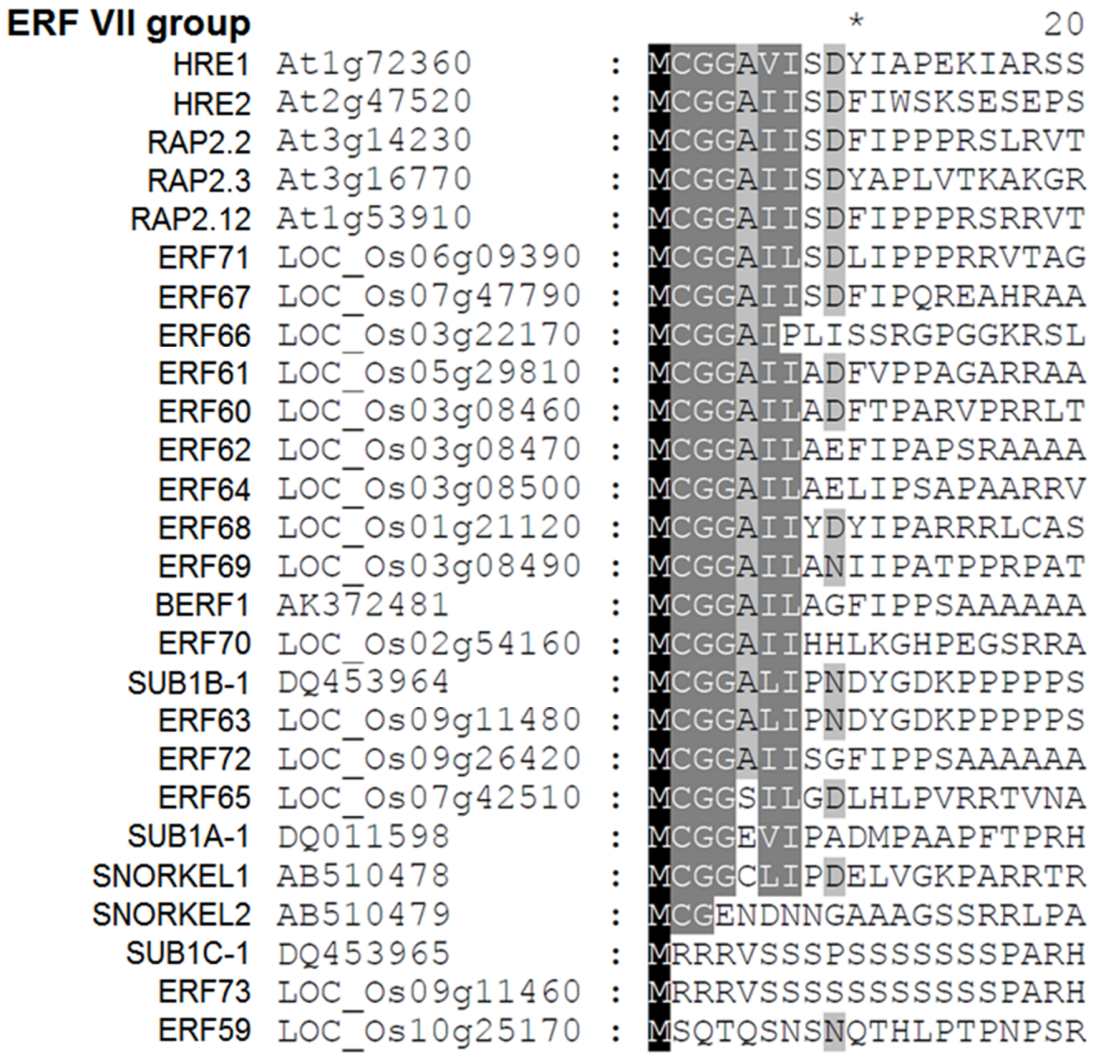

Fig. S6. N-terminal amino acid sequence alignment of group VII ERFs of Arabidopsis and rice.

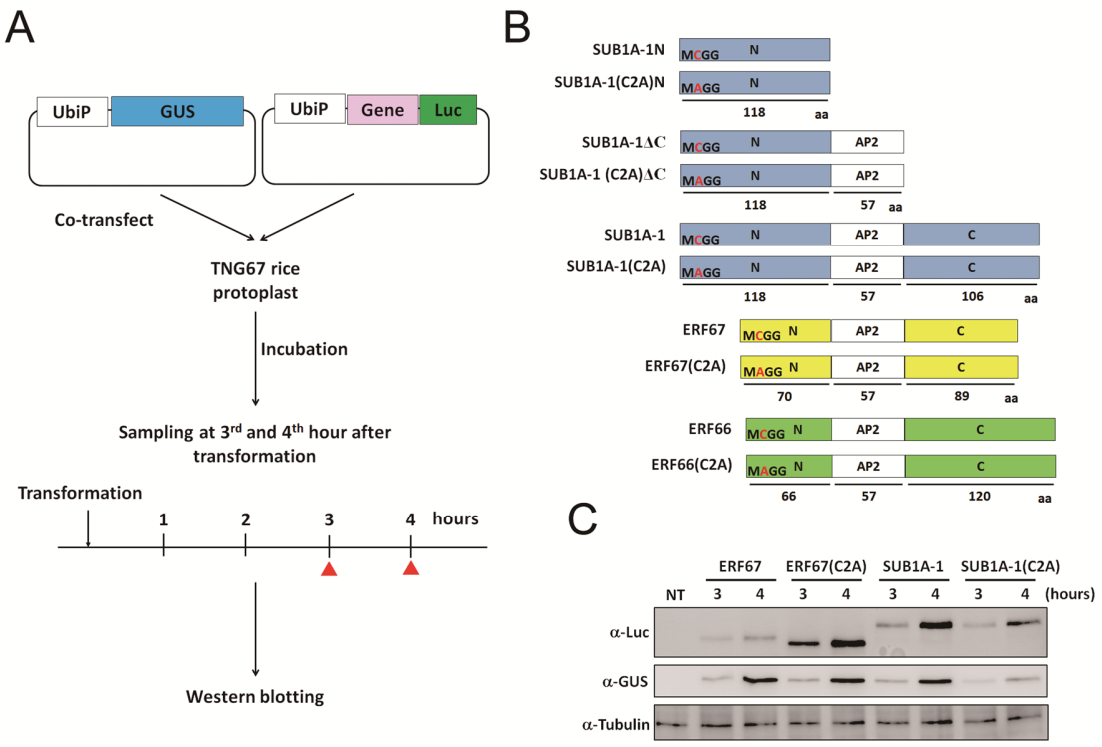

Fig. S7. SUB1A-1 is not a substrate for N-end rule pathway in rice cells. (A) Experimental scheme for protein stability assay in rice protoplast cells. UbiP::GUS plasmid was used as the internal control, and was co-transfected with UbiP::tested gene-Luc into TNG67 rice protoplasts. The transfected protoplasts were incubated in W5 solution for 3 or 4 h then harvested for further western blot analysis. (B) The tested proteins included: truncated SUB1A-1N (13 kDa) and SUB1A-1ΔC (20 kDa), wild-type SUB1A-1 (31 kDa), ERF67 (24 kDa) and ERF66 (26 kDa), and C2A variants, i.e. SUB1A-1(C2A)N, SUB1A-1(C2A)ΔC, SUB1A-1(C2A), ERF67(C2A) and ERF66(C2A). (C) The result of SUB1A-1 and ERF67 protein stability assays. *UbiP::SUB1A/ERF67-Luc* constructs were co-transfected into TNG67 rice protoplasts with a *UbiP::GUS* plasmid, which was used as a stable control. The transfected protoplasts were incubated in W5 solution for 3 or 4 h then harvested for further western blot analyses. NT: Non-transfected.

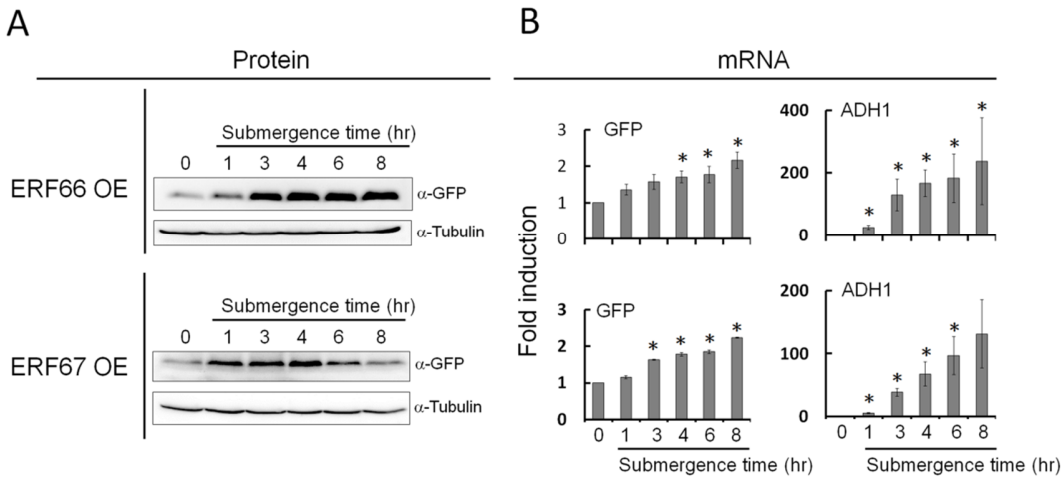

Fig. S8. ERF66 and ERF67 proteins are stabilized *in planta* under submergence. (A) Western blot analyses of protein stability of ERF66 and ERF67 under submergence. Fourteen days old transgenic Arabidopsis seedling expressing GFP-tagged ERF66/ERF67 were treated submergence at room temperature for the indicated times in dark. The time 0 h indicated the seedling samples were taken right before the submergence treatment. (B) Transcript levels of transgenic GFP (27 kDa) tagged ERF67 (24 kDa)/ERF66 (26 kDa) and endogenous ADH were detected by qRT-PCR using GFP and ADH specific primers (Table S1), respectively. Tubulin mRNA was used as the internal control for normalization. The gene expression levels of the samples at 0 h were set to 1 fold. The data represent means±SD from two to three independent replicates. Significant differences compared with the sample at 0 h by Student's *t* test: *, $P < 0.05$

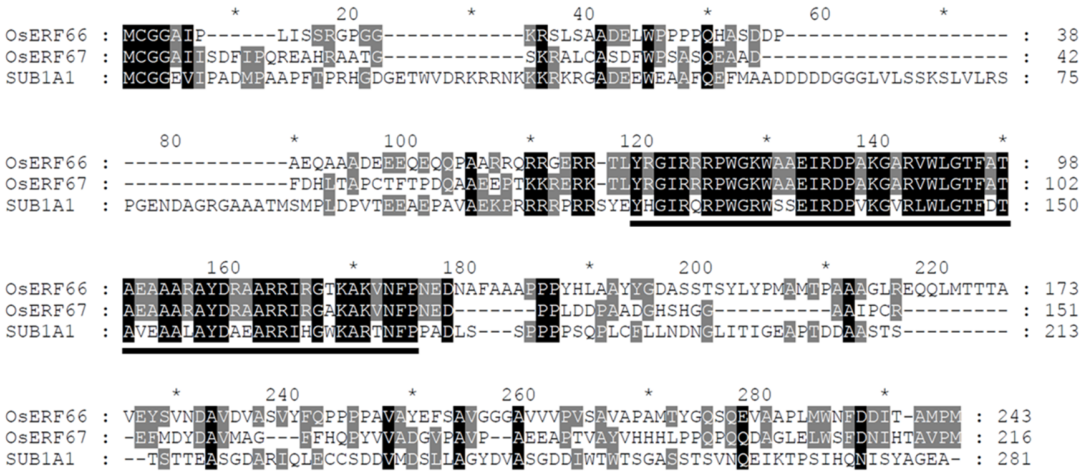

Fig. S9. The protein sequence alignment of SUB1A-1, ERF66 and ERF67. The underline indicates the AP2 domain.

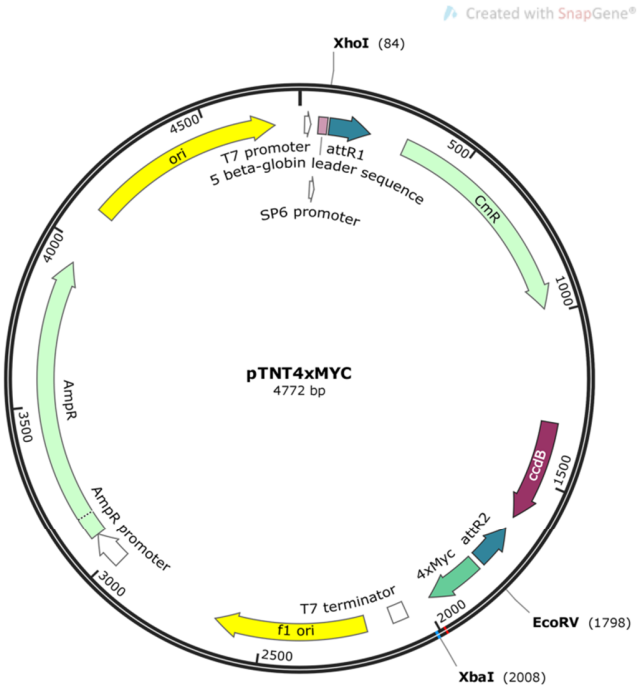


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Fig. S10. The map and sequence of pTNT4xMYC.

Table S1. Primers for qRT-PCR or genomic PCR.

Gene name	Accession	Forward primer	Reverse Primer
ERF59	LOC_Os10g25170	CATGGCATGGCACCTGATGATG	AAACGCATCAAGAACGAGTGACC
ERF60	LOC_Os03g08460	AGACGTCCGTTTGCCTTGCTTG	CTTTGCAGGCTTGCAGCACAAC
ERF61	LOC_Os05g29810	GGGTGTTCTTCTGGGAATCCTTGG	CCTGGCAATGCAATGCTTAACG
ERF62	LOC_Os03g08470	TCGAGCAGTTCGACGATGACTC	GACACGAGATTTGGACGCGAAC
ERF63	LOC_Os09g11480	AGCTCATACTGCTGGAGTGCTG	GCGCGCTGCTAATTAACCATTCC
ERF64	LOC_Os03g08500	ACCCTGTGACTCGATCTTGTACC	GACGAACACATGCTTGCTTGGC
ERF65	LOC_Os07g42510	GGGATTTCGATGTTGATTGCGATG	AGACCGTTCATGTCGGATTCTTGG
ERF66	LOC_Os03g22170	GCTCCGCTCATGTGGAATTTCG	TCCACGGTAAGACAATCACATTGG
ERF67	LOC_Os07g47790	TTCCACCGACTCGTCAGCTTAG	TGCGGGATGAAGTCGGAAATG
ERF68	LOC_Os01g21120	TGTTTCCGCTCCACTTCCCTTC	CGGGATGTAGTCGTAGATGATTGC
ERF69	LOC_Os03g08490	GCGTCATTCGACAGTGACAACAAC	TCGCCGAACAAGAAAGGATCGTC
ERF70	LOC_Os02g54160	TCCTGCAATGAACTCTGCTGCTC	GCAGCCAAATGAGTTGCTTCCC
ERF71	LOC_Os06g09390	CGGCTTCGCTAAAGGTGGATTG	CTCTGATTTCCGCAGCCCATTTG
ERF72	LOC_Os09g26420	CGAAATGTTCTGGTCAGTGTGGTC	CTTGGCCATACACATTCAACATGG
ERF73	LOC_Os09g11460	CGAACAACAGAGCAGATTCAGG	TTAGGCGAGTCGCATGTCAAGG
Sub1A	DQ011598	ACAACGGCCTCATCACAATC	AGGCTCCAGATGTCCATGTC
Tubulin	LOC_Os07g38730	CGCAGTTGCAACCATCAAGACG	ACTTGAATCCAGTAGGGCACCAG
GFP tag		CGACCACTACCAGCAGAACACCC	CAGCTCGTCCATGCCGAGAGT
ADH1	At1g77120	CATGAACAAGGAGCTGGAGCTTG	CTCTCCCTTCAGCATGTAATCAAAGG
TUB3	At5g62700	GGTTGGTTTTGCTCCTCTCACC	TAGCGTCCGTGCCTTGGGTC
GST tag		TTTGTATGAGCGCGATGAAG	ACGCTCTTTTGGACAACCAC
For ChIP-qPCR			
ERF66 promoter		CCTCGCTCTCCTCGTCACCG	GTGGGTGCGGTCCGCGTAGG
ERF67 promoter		CCCCTCTCCTTTTACACACG	GAGCCGTGGGCCGCGGCTGG
For hygromycin genomic PCR			
Hyg		TGCATCGGCCGCGCTCCCGATTCCGG	CCACGGCCTCCAGAAGAAGATGTTGGCG

Table 2. Primers for cDNAs cloning.

Gene fragment	Forward primer	Reverse primer
For trans-activation assay and *in vitro* stability assay		
SUB1A-1/2	ATGTGTGGAGGAGAAGTGATCCCCGC	TCAGGCTTCCCCTGCATATGATATGTTTTG
SUB1A-1/2 (C2A)	ATGGCGGGAGGAGAAGTGATCCCCGC	TCAGGCTTCCCCTGCATATGATATGTTTTG
ERF66 promoter	TGACACGCTTGGGGGCATGCGGTAGC	ATCTATCTTCACTAGCTAGCTCGATCG
ERF67 promoter	GAAATCTATCAAAACATTGTGCTATTCTTC	CTTTGCTGCTGCTGCGCGCTTGCGG
For protein stability assay		
SUB1A-1	CGCGGATCCATGTGTGGAGGAGAAGTGATCCCCGC	CCCAAGCTTGGCTTCCCCTGCATATGATATGTTTTG
SUB1A-1(C2A)	CGCGGATCCATGGCGGGAGGAGAAGTGATCCCCGC	CCCAAGCTTGGCTTCCCCTGCATATGATATGTTTTG
SUB1A-1 N	CGCGGATCCATGTGTGGAGGAGAAGTGATCCCCGC	CCCAAGCTTCCGCCTCGGCCGGCGCCGGCGAGGCTTC
SUB1A-1(C2A)N	CGCGGATCCATGGCGGGAGGAGAAGTGATCCCCGC	CCCAAGCTTCCGCCTCGGCCGGCGCCGGCGAGGCTTC
SUB1A-1 ΔC	CGCGGATCCATGTGTGGAGGAGAAGTGATCCCCGC	CCCAAGCTTTGGGAAGTTTGTCCGGGCTTTCCAGCC
SUB1A-1(C2A)ΔC	CGCGGATCCATGGCGGGAGGAGAAGTGATCCCCGC	CCCAAGCTTTGGGAAGTTTGTCCGGGCTTTCCAGCC
ERF66	CGCGGATCCATGTGCGGCGGCGCCATCCCGCTG	CCCAAGCTTCATTGGCATGGCCGTGATGTC
ERF66(C2A)	CGCGGATCCATGGCGGGCGGCGCCATCCCGCTG	CCCAAGCTTCATTGGCATGGCCGTGATGTC
ERF67	CGCGGATCCATGTGTGGCGGCGCGATCATTTCCGAC	CCCAAGCTTCATCGGCACGGCCGTGTGG
ERF67(C2A)	CGCGGATCCATGGCGGGCGGCGCGATCATTTCCGAC	CCCAAGCTTCATCGGCACGGCCGTGTGG
Luc	CCCAAGCTTGAAGACGCCAAAAACATAAAG	CGCGGATCCTTACAATTTGGACTTTCCGCCCTTC
For recombinant protein expression		
SUB1A-1	CCGCCGGATATCAGAGAACCTCTACTTCCAATCGATGTGTGGAGGAGAAGTGATCCCCG (the TEV protease cleavage site is underlined)	CCGCCGCTCGAGGGCTTCCCCTGCATATGATATGTTTTG
SUB1A-1N	CCGCCGGATATCAGAGAACCTCTACTTCCAATCGATGTGTGGAGGAGAAGTGATCCCCG (the TEV protease cleavage site is underlined)	CCGCCGCTCGAGCCGCCTCGGCCGGCGCCGGCGAGGCTTC
For pTNT4xMYC construction		
ccdB fragment	CCGCTCGAGACAAGTTTGTACAAAAAAGCTGAA (the XhoI site is underlined)	CCGGATATCCACCACTTTGTACAAGAAAGCTGAA (the EcoRV site is underlined)
4xMYC fragment	CCGGATATCGATAACAGCGGGTTAATTAACGG (the EcoRV site is underlined)	CTAGTCTAGACGATCGGGGAAATTCGAGCTCTAAGC (the XbaI site is underlined)

Table S3. Probes for EMSA.

Probe	sequence
ATCTA reference probe (59)	CAATCTAAATATCTAAAATATAAA
GCC reference probe (60)	CGCGATCGCCGCCGCCGGATCGCG
SUB1A-1 GCC1	TGGAGAGGGCCGCCCCATCGTG
ERF66 GCC1	GATCGATCGCCGCCGGTTTAGA
ERF66 GCC2	TAGATCTAGGCGGCGACCATGG
ERF66 GCC3	GGTGTCGTGGCGGCGGGGTCGA
ERF67 GCC1	CCCCCCGCGCCGCCACGCTCT
ERF67 GCC2	AAAGCAGCGGCGGCACCGAGCG
ERF67 GCC3	GAGCAGCAGCCGCCGTCACCAT
ERF67 GCC4	AGCCGGCAGCCGCCCGTGTCCA

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