國立臺灣大學生命科學院植物科學研究所

碩士論文

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Master Thesis

水稻 ERFVIIs 對於淹水反應的功能性分析

Functional analysis of ERFVIIs in rice under Submergence stress

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國立臺灣大學碩士學位論文
口試委員會審定書

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本論文係 曾瓊瑜 君(學號 R09B42003)在國立臺灣大學植物科學研究所完成之碩士學位論文，於 2022 年 7 月 12 日承下列考試委員審查通過及口試及格，特此證明。

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短暫的研究生涯很快的畫下句點，回首這兩年的時光有多少蹉跎、又有多少進步?感謝兩年前的自己選擇了一位好老師並且來到中研院做實驗，雖然施老師希望我自己摸索，但很多時候我會迷失了方向，而老師就像一盞燈，每次給予我的指引都有獨到見解，並且會細心的改正我的小錯誤，也很感謝師母一直帶著我做實驗，老師和師母總是給我鼓勵與關心，讓我能正向地面對研究結果。雖然實驗室沒有同齡人，但是學長姐的諸多提點使我能少走許多彎路，很感謝致成學長與美怡學姐，提供了材料與技術上的支持，幫助我解決了許多實驗上的問題，並且教導我解釋結果的方向與細節；感謝保元學長，除了給予我實驗上的幫助外，相仿的研究成果奠定了我的研究基礎；感謝崑縉學長、銹銀學姐與婉潔學姐，在進度報告時給予我充分的回饋，兩位學姊也將實驗室管理的井井有條。雖然我在台大的時間比較短，但很感謝植科所的教授們在課堂上所提供的建議，使我能更全面的討論實驗成果。此外，特別感謝林教授與謝教授，仔細地審查論文內容並提供了許多撰寫的建議，使本篇論文能更加客觀與嚴謹。最後，感謝我的家人朋友們對我的支持與包容，由於路程遠、只有逢年過節才會返鄉，每當回家就顯得近鄉情怯，但家人朋友們會盡可能滿足我的口腹之欲和傾吐欲，降低我隻身離家的孤寂之感。

一場 Covid-19 疫情佔據了我碩士班一半的日子，並且還在持續中，變了調的生活使的人與人之間多了一份安全距離，所幸的是，疫情流行趨勢的變化與疫苗的普及預告著正常生活的回歸，感謝這場疫情中默默守護的人們，使許多無辜的民眾能幸免於難，但願能在最少的哀悼下結束這場災難。

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中文摘要

隨著氣候變遷，極端的天氣現象越發嚴重與頻繁，而劇烈降雨所造成的淹水事件會造成植物浸沒於水中，進而影響植物生長與生存。第七類乙烯轉錄因子(The group VII of ethylene response factors, *ERFVIIs*)可以作為氧氣感應者，其 N 端保守序列可以作為 N-end rule 的受質，使其蛋白質在一般環境下不穩定而降解，而在淹水以及低氧環境下，穩定的蛋白質能協調下游基因以降低淹水所造成的傷害。水稻對於淹水環境有著天然耐受性，但只有少數品系在完全浸泡於水中超過 2 週後能恢復生長。水稻帶有 18 個 ERFVII 基因，其中的 *SUB1A-1* 作為重要調控因子，只存在於特定品系，可以調控許多代謝反應並抑制植物生長，降低能量負擔。此外，SUB1A-1 可以調控 *ERF66* 和 *ERF67* 表現，可共同促進淹水耐受性。除了 SUB1A 相關機制外，水稻 ERFVIIs 對於淹水耐受性的調控是未知的。

本篇報導透過轉錄量分析，發現數個基因(*ERF65*、*ERF70*、*ERF71* 與 *ERF72*)持續表現，並且在不同遺傳背景下亦然，代表其功能可能是保守並且與淹水時快速反應有關。透過在水稻原生質體暫時表達，ERF65、ERF70 和 ERF72 可以激活 *ERF67* 啟動子，而 ERF65/70/72 與 SUB1A 的共表達可以促進 *ERF67* 的轉錄，多個 ERFVII 蛋白佔據於 *ERF67* 啟動子上的 GCC box 可能是造成共協調的主因。有趣的是，雖然 SUB1A-1 與 SUB1A-2 均可與 ERF65/70/72 共同激活 *ERF67* 表現，但 SUB1A-2 無法有效誘導內生的 *ERF67* 表現，這突顯了 SUB1A 的磷酸化在調控功能上的重要性。此外，ERFVIIs 可以調控非共生血紅蛋白(non-symbiotic hemoglobin, *nsHBs*)的表現，其作為一氧化氮清除者，在缺氧期間可調節細胞內一氧化氮含量。ERF65、ERF70 與 ERF71 有較高能力促進 *HB1* 和 *HB2* 表現，ERF72 為中等，SUB1A-1 與 ERF67 則有較低能力促進 *HB2* 表現，而當 ERF67 與 ERF71 共表達時，可能產生競爭關係而微調 *HB2* 表現量。

總之，我們提出了一條 ERFVII 的調控路徑，有助於水稻對於淹水的基礎耐受性；而在耐淹水品系中，SUB1A 的參與可以增加對 *ERF67* 的調控，進而延長浸沒的生存時間。

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關鍵字: 淹水耐受性；水稻；轉錄調控；乙烯轉錄因子；非共生血紅蛋白

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Abstract

With climate change, extreme weather phenomena are more severe and frequent. Flooding events by severe rainfall cause plants to be submerged in water, which will affect the growth and survival of plants. The group VII of ethylene response factors (*ERFVIIs*) are the substrates of N-end rule which make their protein unstable under normal oxygen conditions. Under submergence and low oxygen, the stabilized ERFVII proteins could coordinately regulate downstream genes to reduce the damages caused by flooding. Rice (*Oryza sativa)* is naturally tolerant to flooding, but only a few cultivars can survive after fully submerged in water for a prolonged period of time. There are eighteen ERFVIIs in rice. *SUB1A-1* that only exists in specific cultivars is a master regulator to coordinate metabolic responses and repress plant growth during submergence which can reduce energy burden. In addition, SUB1A-1 could regulate the expression of *ERF66* and *ERF67*, which could promote flooding tolerance. Except for SUB1A-dependent mechanisms, the regulation of *ERFVIIs* in rice for flooding tolerance is unknown.

In this study, several ERFVII genes including *ERF65*, *ERF70*, *ERF71* and *ERF72* were found constitutively expressed in different genetic backgrounds via transcription analysis, indicating their function might be conserved and associated with rapid response to flooding. Through transient expressions in rice protoplasts, ERF65, ERF70 and ERF72 could activate the promoter of *ERF67*. Co-expression of ERF65/70/72 with SUB1A could enhance the transcript of ERF67. The occupancy of GCC boxes on the *ERF67* promoter by multiple ERFVII proteins might be the main reason for synergistic transcriptional activation. Interestingly, SUB1A-1 and SUB1A-2 respectively with

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ERF65/70/72 could co-activate the expression of the *ERF67-Luc* reporter gene, but the group of SUB1A-2 failed to efficiently induce the endogenous *ERF67* expression. It highlighted the importance of the phosphorylation of SUB1A for the regulatory function. In addition, ERFVIIs could regulate the expression of non-symbiotic hemoglobins (*nsHBs*), as nitric oxide (NO) scavengers to modulate NO content during hypoxia. ERF65, ERF70 and ERF71 had better capabilities to activate the expression of *HB1* and *HB2*. SUB1A-1 and ERF67 showed low abilities to induce the expression of *HB2*. Co-expression of ERF67 and ERF71 might compete for the binding sites and fine-tune the expression of *HB2*.

In summary, we proposed a regulatory pathway of *ERFVIIs* which contributed to the basal tolerance of flooding for rice. In tolerant cultivars, the involvement of *SUB1A-1* would enhance the expression of *ERF67*, which would prolong the survival during submergence.

Key words: submergence tolerance; rice; transcription regulatory; ethylene response factors; non-symbiotic hemoglobins

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Chapter 1: Background and knowledge

1.1 Flooding is a worldwide stress

According to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC), climate change is already affecting weather and extreme climates across the globe, such as heavy precipitation and droughts. The frequency and intensity of heavy precipitation events have increased since 1950s over most land areas, contributing to increase in severe flooding events. Flooding cause life loss and economic loss, especially in the agriculture sector. Plants would partially or entirely immerse in water during flooding, which influences the growth and survival of plants. Reduction of gas diffusion rate in water limits the uptake of oxygen (O_2) and affects mitochondrial respiration, and reduced light intensity in turbid flashwaters inhibits underwater photosynthesis (Tamang and Fukao, 2015). Prolonged flooding caused plants energy crisis and continuous anaerobic metabolism would cause the accumulation of phytotoxic end-products (Bailey Serres and Voesenek, 2008). After submergence, plants suddenly expose to aerobic conditions and encounter dehydration stress, oxidative damage and photoinhibition (Fukao et al., 2011). In addition, floods increase the probability of pathogen infection and disease transmission. Taken together, plants would suffer from complex stresses under flooding events and how to reduce the damage is an important issue.

1.2 Strategies for rice to adopt flood stress

Rice (*Oryza sativa)* is a semiaquatic plant that is well adapted to partial flooding. Most rice varieties could promote underwater elongation, but deep flooding that cause

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plants complete submergence in water would exhaust plant energy and cause death within a matter of days (Bailey Serres et al., 2010). A limited number of rice varieties can escape from a slow progressive flooding through rapidly elongation of underwater stem (escape strategy) or tolerate a deep transient flash flooding through restriction of growth and metabolism (quiescence strategy) (Bailey Serres et al., 2012). For escape strategy, deepwater rice varieties increases height and keeps up with water level to adapt to the rainy season for months. Two ethylene response factor (ERF) family genes, *SNORKEL1 (SK1)* and *SNORKEL2 (SK2)*, were induced by ethylene and promoted internode elongation via gibberellin (GA) production to outgrow rising flood water and ensure O_2 supply (Hattori et al., 2009). For quiescence strategy, *Submergence 1* (*SUB1*) locus which encodes a cluster of two or three subgroup VII of ERF (ERFVIIs) was shown to confer the ability of completely submergence tolerance. All rice accessions contained *SUB1B* and *SUB1C*, and *SUB1A* was limited to some *Indica* and *aux* varieties (Xu et al., 2006). *SUB1A-1*, a tolerance-specific allele, is a major determinant of submergence tolerance. *SUB1A-1* increased the accumulation of GA signaling repressor, *Slender Rice-1* (*SLR-1*) and *SLR1 like-1* (*SLRL-1*), limiting GA-mediated shoot elongation and carbohydrate degradation, and thereby reduced energy consumption (Fukao and Bailey Serres, 2008). Although the above genes had opposite functions in regulating plant growth, their contributions to flooding tolerance justified more molecular studies of *ERFVIIs*.

1.3 The role of group VII ethylene response factors (ERFVIIs) in flooding stress

The group VII of ERF transcription factors (*ERFVIIs*) is involved in a wide range of physiological processes, such as plant growth, development and stress response.

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ERFVII contains single DNA-binding APETALA2(AP2)/ERF domain and several other motifs, including N-terminal (Nt)-MCGGAII/L, which is conserved in all flowering plants and functions as an N-degron, rendering these proteins the substrates of the N-end rule pathway (Gibbs et al., 2015). The N-end rule pathway is a proteolysis system that the substrate protein containing destabilizing residue at N-terminal is recognized by an E3 Ubiquitin ligase and then degraded by proteasome (Dissmeyer, 2019). The degradation process includes a series of modification on the substrate proteins by diverse enzymes. In the case of ERFVIIs, the Nt-Met of substrate protein is cleaved by MET AMINOPEPTIDASE (MetAP) to expose a tertiary destabilizing Nt-Cys residue. In the presence of O_2 or nitric oxide (NO), the Nt-Cys is susceptible to oxidation and would be converted to Cys-sulfonic acid, which permits an Arg attachment by Arg-tRNA TRANSFERASES (ATEs) to generate a primary destabilizing Nt-Arg residue (Dissmeyer, 2019). The Nt-Arg-Cys is then recognized by N-recognin E3 ligase PROTEOLYSIS6 (PRT6), which targets protein to degradation via polyubiquitination.

There are five members of *ERFVIIs*, *HRE1*, *HRE2*, *RAP2.12*, *RAP2.2* and *RAP2.3* in Arabidopsis, all the which are the substrates of the N-end rule pathway *in vitro* and function to sense O_2 (Gibbs et al., 2011). Under low O_2 (hypoxia), the proteins accumulate to coordinate the transcriptional responses to O_2 limitation. After de-submergence, the degradation of ERFVIIs could be a signal to switch off the hypoxia response and recover growth (Licausi et al., 2011). The transcript levels of *HRE1* and *HRE2* are induced by hypoxia while *RAP2.12*, *RAP2.2* and *RAP2.3* were constitutively expressed and slightly changed during hypoxia (Hinz et al., 2010; Yang et al., 2011; Gasch et al., 2016). Overexpression of *HRE1* could increase the induction of several anaerobic genes under hypoxia while *hre1hre2* knockout mutant showed decreased

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expressions in the same genes and lower tolerance to hypoxia (Licausi et al., 2010). RAP2.2, RAP2.12 and RAP2.3 had strong activation ability of anaerobic promoters and mutation of each gene would have decreased the submergence tolerance (Bui et al., 2015; Papdi et al., 2015).

1.4 The roles of ERFVIIs in rice

In rice, there are eighteen members of *ERFVIIs* and some of them are cultivar-specific, including *SUB1A*. The expression levels and alleles in different cultivars were related to submergence tolerance. *SUB1A-1* from tolerant cultivar FR13A was highly induced during submergence while *SUB1A-2* from sensitive cultivar IR29 had lower expression (Lin et al., 2019). The difference between these alleles was a substitution from serine (SUB1A-1) to proline (SUB1A-2) at 186th amino acid residue of the SUB1A protein, which influenced the phosphorylation of SUB1A by MPK3 (Singh and Sinha, 2016). The activity of MPK3 during submergence was dependent on SUB1A genotype, but how SUB1A regulates it was unknown. It is also unclear how phosphorylation affects the ability of SUB1A to regulate the expression of downstream genes. SUB1A-1 could directly regulate the expression of two *ERFVIIs*, *ERF66* and *ERF67*, and overexpression of *SUB1A-1*, *ERF66* or *ERF67* in sensitive TNG67 could improve the submergence tolerance (Lin et al., 2019). Interestingly, despite having the conical N-degron sequences MCGG, SUB1A-1 could escape from N-end rule through self-interaction and shielding the N-degron, which influenced the protein stability after de-submergence (Lin et al., 2019). *SUB1A* could coordinate physiological and molecular response to cellar water deficit following de-submergence (Fukao et al., 2011). It was unknown how SUB1A mediates the opposite pathways during de-

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submergence. Although *SUB1A* is important for submergence tolerance of rice, ectopic expression of *SUB1A* in Arabidopsis would reduce the survival of submergence (Peña Castro et al., 2011). Except for *SUB1A*-dependent mechanisms, the studies of *ERFVIIs* in rice were little.

1.5 The regulation of ERFVIIs on expression of the target genes

The regulation of transcription factors on expression of the target genes is a complex process determined by many components, including protein-DNA interaction. ERF proteins are well-known to bind the GCC box element (5'-GCCGCC-3') which is present in the promoters of many Pathogenesis-related (*PR*) genes. Structural analysis showed that the conserved AP2 domain of AtERF1 could contact the DNA base (Allen et al., 1998). In addition, the DRE/CRT element with the core sequence 5'-A/GCCGAC-3' in the promoters of many dehydration-induced genes was specifically bound by DREB proteins containing an AP2/ERF domain (Sakuma et al., 2002). Some studies showed the interactions between ERFVIIs and DNA. For example, SUB1A-1 could bind the GCC boxes in the promoters of *ERF66* and *ERF67* (Lin et al., 2019). The GCC boxes from *ERF66/67* promoter containing different flanking sequence would affect the binding affinities of SUB1A-1 to the GCC boxes. HRE2 could bind to the GCC box element and DRE/CRT element *in vitro* (Lee et al., 2015). RAP2.2 and RAP2.12 could bind the hypoxia-response promoter element (HRPE) with the consensus sequence 5'-AAACCA(G/C)(G/C)(G/C)GC-3', which is necessary for transactivation of two hypoxia-response genes, *LBD41* and *PCO1* (Gasch et al., 2016).

1.6 The role of non-symbiotic hemoglobin (nsHBs) during hypoxia stress

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Non-symbiotic hemoglobins (nsHBs), which are heme-containing proteins, are involved in plant development and stress response. The heme group is able to bind diatomic gasses of biological relevance such as O_2 and NO. Two classes of *nsHBs* can be distinguished based on the phylogenetic analysis and expression patterns (Trevaskis et al., 1997). The expression levels of class 1 nsHBs (*nsHB-1s*) were induced by low O_2 treatment. As nsHB-1s displayed high affinities and lower dissociation to O_2, they might not function as O_2 transporters (Trevaskis et al., 1997). nsHB-1 could function as NO dioxygenase and contribute to maintaining energy state. Under hypoxia, NO is converted to NO_3^- by nsHBs and this process is accompanied with oxidation of NAD(P)H, which replenishes the pool of NAD^+ for continued fermentation (Igamberdiev, 2004). Overexpression of *AHB1* in Arabidopsis would enhance the survival during hypoxia, and *AHB1*-silenced lines would increase the emission of NO (Hunt et al., 2002; Hebelstrup et al., 2012). A recent study showed that *AHB1* was induced by ethylene, which depleted NO and stabilized RAP2.3 under normoxic conditions (Hartman et al., 2019). The expression of *AHB1* was induced by the RAP2.2, RAP2.12 and RAP2.3 through trans-activation assays (Bui et al., 2015). These studies indicated that nsHBs and ERFVIIs might form a regulatory loop to modulate hypoxia response.

In rice, there are five *nsHBs* and many studies focused on the *HB1* and *HB2*. The expressions of *HB1* and *HB2* were induced by nitrate, nitrite and NO donor using rice culture cells (Ohwaki et al., 2005). Previous studies showed that several hormone-response elements existed in the promoters of *HB1* and *HB2*, and that *HB2* was induced by cytokinin using transient assays (Ross et al., 2004). Although there are GCC boxes in the promoter of *HB2*, little evidence showed *HBs* in rice could respond to abiotic stress

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and were regulated by ethylene or ERF proteins. RNA-sequence data showed that in *SUB1A-1*- OE and *ERF67*-OE lines, the expressions of *HB1* and *HB2* were up-regulated during 24h submergence (Lin et al., 2019), but it should be confirmed whether ERFVIIs could directly regulate the expressions of *nsHBs*.

To determine the potential roles of ERFVIIs in response to submergence, we investigated the transcriptional patterns of *ERF*s in different genetic backgrounds and found that several *ERFVIIs*, including *ERF65*, *ERF70*, *ERF71* and ERF72, had constitutive expressions under normoxia and hypoxia. Through trans-activation assays in TNG67 protoplasts, these ERFVIIs displayed different capabilities to activate the expressions of *ERF67* and *nsHBs*. Furthermore, ERF65, ERF70 and ERF72 would cooperate with SUB1A-1 to enhance the expression of *ERF67*. Through confirming the binding sites, various ERFVIIs occupied at the *ERF67* promoter would partially contribute to synergistic transcription.

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Chapter 2: Material and methods

2.1 Growth Conditions and submergence treatment

Rice seeds were sterilized with 70% ethanol for 1 min, 1.5% sodium hypochlorite containing 0.1% Tween 20 for 25 min, and then washed with sterilized water at least five times until no visible bubbles. The sterilized seeds were placed on wet filter paper in Petri dishes at 37°C in the dark for 4 d. After incubation, the germinated seeds were transferred onto an iron grid in a beaker containing Kimura B solution [0.091 mM $(NH_4)_2SO_4$, 0.046 mM KNO_3, 0.137 mM $MgSO_4$, 0.046 mM KH_2PO_4, 0.015 mM Fe-citrate, 0.091 mM $Ca(NO_3)_2$, 1M HCl, 0.00125 mM H_3BO_3, 0.0001 mM $MnSO_4$, 0.0001mM $ZnSO_4$, 0.00003 mM $CuSo_4$, 0.00002 mM H_2MoO_4, pH5.7], and the solution was renewed per 2 days. For protoplast preparation, the hydroponically seedlings were grown in a growth chamber at 28 °C in the dark for 7 d.

For submergence treatment, the seedlings were grown in a growth chamber at 28 °C with a 16-h-light (120–125 $\mu mol \cdot m^{-2} \cdot s^{-1}$)/8-h-dark cycle until they were 14-d-old. A water tank (40 cm long, 40 cm wide and 70 cm tall) was filled with tap water to 55 cm high and incubated overnight to balance oxygen concentration. Then, the beakers containing 14-d-old plants were placed into the water tank at 28 °C in the dark. The shoot tissues were harvested at the indicated times and frozen in liquid nitrogen for RNA extraction.

2.2 RNA extraction and reverse transcription

TRIZOL reagent (Invitrogen) was used to isolate total RNA from treated samples. For shoot sample, the sample was ground with liquid nitrogen and 600 μL of TRIZOL

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was added into the powder sample. For protoplast sample, 1000 μL of TRIZOL was added after W5 solution was removed. The samples were vortexed and placed at room temperature for 5 min. Chloroform (1/5 of TRIZOL volume) was added and shaken vigorously. After incubating at room temperature for 3 min, the samples were centrifuged at 12,000 ×g for 15 min at 4°C. The supernatants were transferred into a new tube and centrifuged again for 10 min. The supernatants were collected and isopropanol (1/2 of TRIZOL volume) was used for precipitate RNA. After incubation at room temperature for 10 min, the samples were centrifuged at 12,000 ×g for 15 min at 4°C and the supernatants were removed. The pellets were washed with 1 ml 70% ethanol twice and followed by 1 ml 100% ethanol. The pellets were centrifuged at 12,000×g for 15 min at 4°C when washed. The pellets were airdry and then dissolved in RNase free water. The samples were heated at 55°C for 5 min to completely dissolve the pellets. The contaminating DNA was removed by TURBO DNA-free kit (Ambion). DNase buffer and DNase I were added to the RNA extraction and mixed well. After incubation at 37°C for 30 min, the mixtures were centrifuged at 12,000 ×g for 2 min at 4°C. The supernatants were transferred to a new tube and then centrifuged again. The supernatants were collected and the concentration was detected by Nanodrop spectrophotometer.

For reverse transcription, 2 μg of total RNA was added with 1 μg oligo-dT and 1 μL 10 mM dNTP. Mixtures were denatured at 70°C for 10 min followed by cooling down on ice for 5 min. 8 μL of reverse transcription mix [4 μL 5X first strand buffer, 2 μL 0.1M Dithiothreitol (DTT), 0.5 μL ribonuclease inhibitor (RNase OUT), 0.5 μL RNase free water, and 1 μL RNA Moloney murine leukemia reverse transcriptias (M-MLV RT)] was added and mixed well. The mixtures were incubated at 37°C for 1 h and

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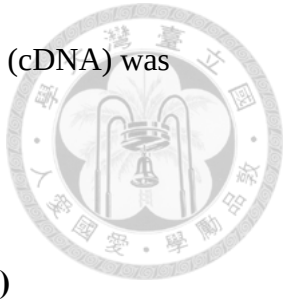
then 65°C for 10 min for heat inactivation. The complementary DNA (cDNA) was diluted with 80μL ddH_2O and stored at -20°C.



2.3 Quantitative real-time polymerase chain reaction (qRT-PCR)

One μg cDNA was mixed with 10 μL SYBR Green PCR Master Mix, 0.4 μM of forward and reverse gene specific primers. qRT-PCR was performed on a Bio-Rad CFX Conncet ™ Real Time System and applied the following condition: 50°C for 2 min, followed by 40 cycles of 95°C for 10 min, 95°C for 15 sec and 60°C for 1 min. The melting curve was measured under the default condition. The expression level of *tubulin* was used as an internal control. Primer sequences was listed in Table 1.

2.4 Plasmid construction

The coding sequences (CDS) of *ERF65*, *ERF67*, *ERF70*, *ERF71* and *ERF72* were amplified by PCR using submerged TNG67 cDNA as the template, and the CDS of *SUB1A-1* and *SUB1A-2* were respectively from submerged FR13A and IR29 cDNA. The DNA fragments were ligated into the pCR8/GW/TOPO (Invitrogen), and then subcloned into pGWB417 and pET32a by the Gateway system (Invitrogen). The 1.2, 1.09, 0.47 and 0.39-kb upstream promoter sequence of *ERF67* were amplified by PCR from the genomic DNA of TNG67 and ligated into the pCR8 vector followed by subcloning into pGW-RenLuc. Mutation of the GCC box in the promoter sequence of *ERF67* were generated by PCR-based site-directed mutagenesis (Zheng, 2004). The 1.5-kb upstream promoter sequence of *HB1* and *HB2* were amplified by PCR from the genomic DNA of TNG67 and ligated into the pCR8 vector. The plasmids were digested

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with TpnI and SmaI, and the promoter regions were ligated into TpnI and SmaI-digested pGreenII 0800-Luc. Primer sequences was listed in Table 2.

2.5 Protoplast preparation and transformation

For protoplast preparation, the stems and sheaths of 7-day-old TNG67 seedlings were cut into 0.5-mm strips and incubated in the enzyme solution [2% Cellulase RS (Yakult), 1% macerozyme R10 (Yakult), 10mM MES, pH 5.6, 0.6 M Mannitol, 10 mM $CaCl_2$, and 0.1% BSA]. The mixtures were shaken in dark at 40 rpm for 3 h and then shaken at 80 rpm for 30 min. After digestion, protoplasts were released by filtering through 50-μm nylon meshes and using W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH5.6, and 5 mM glucose) to wash the strips. The protoplasts were collected by centrifugation at 250 ×g for 3 min with a swinging bucket. The supernatant was removed and the protoplast pellets were resuspended in W5 solution. This step was repeated twice and the resuspended protoplasts were put on ice for at least 30 min. After centrifuged at 250 ×g for 3 min and removed the W5 solution, the MMG solution (0.6 M mannitol, 15 mM $MgCl_2$, and 4 mM MES, pH5.6) were used to resuspended the protoplasts to a final concentration of 2×10^6 per milliliter.

For protoplast transformation, a total of 4×10^5 protoplasts in 0.2 ml MMG solution were mixed with 10-20 μg of plasmid and put on ice for 10 min. Then, equal volume of PEG-calcium solution [40% PEG 4000 (95904; Sigma-Aldrich), 0.6 M mannitol, and 0.1 M $CaCl_2$] was added, and the mixture was incubated at room temperature for 20 min. Then, 3 mL of W5 solution was added and gently mixed. The protoplasts were centrifuged at 250 ×g for 3 min and the supernatant was removed. The washing step by

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W5 solution was repeated twice. The protoplasts were resuspended gently in 1 mL W5 solution and transferred to a 1.5 mL microfuge tube.

2.6 *Trans*-activation assay

TNG67 rice protoplasts were co-transformed with the effector, reporter and internal control plasmids at a mass ratio of 1:1:0.1. After incubated in dark for 15 h, the protoplasts were collected by centrifugation for 15 s at 13,000 rpm and the supernatant was removed. The protoplasts were resuspended in 60-100 μL of 1X passive lysis buffer (Promega) and vortexed for 1 min at high speed. The disrupted protoplasts were centrifuged at 13,000 rpm and 4°C for 10 min. The supernatant was collected and 10-20 μL sample was used for luciferase activity assay. The luciferase activity was analyzed by using a microplate reader and Dual-Luciferase Assay System (Promega) following the manufacturer's instructions.

2.7 Protein expression and purification

The plasmids pET32a-ERF65, pET32a-ERF70, pET32a-ERF72 or pET32a-SUB1A-1 were transformed to *Escherichia coli* Rosetta (DE3). Recombinant protein expression was induced at O.D 0.6 by adding 0.25-0.5mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 25 °C for 6 h and the cells were harvested by centrifugation at 3,000 ×g at 4 °C for 15 min. Cells were washed with lysis buffer (25 mM Tris, pH 8.0, 300mM NaCl and 10mM Imidazole) twice and resuspended in lysis buffer supplemented with Benzonase, 1 mg/mL of Lysozyme, and Protease Inhibitors. After sonicated, the lysate was centrifuged at 10,000 ×g for 15 min. The supernatant was added into a column packed with NiNTA resin pre-equilibrated with lysis buffer.

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The column was washed with 10 column volumes of wash buffer (25 mM Tris, pH 8.0, 300 mM NaCl, and 20 mM-100 mM imidazole). Proteins were eluted with the elution buffer (25 mM Tris, pH 8.0, 300 mM NaCl, and 200 mM Imidazole). The protein concentrations were measured by Bio-rad Protein Assay following the manufacturer's instructions.

2.8 Electrophoretic mobility shift assay (EMSA)

Fluorescein amidite (FAM) labeled DNA probes were synthesized by PURIGO Biotechnology Co., Ltd and the sequence are listed in Table 3. DNA-protein binding reaction was carried out by incubation of 0.2 μM of FAM-labeled probe with 0.5 μM of recombinant proteins 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, 1.2 mM dithiothreitol (DTT), and 0.5 μg of poly(dI-dC) (Sigma). The mixtures were incubated at room temperature for 20 min. Protein-DNA complexes were separated on a 6% native polyacrylamide gel and the FAM signal was imaged using a Typhoon Scanner (GE Healthcare).

The label-free DNA probes were amplified by PCR using specific primers listed in Table 2. The plasmids of pERF67-RenLuc and pERF67-gcc12m-RenLuc were used as the PCR templates. DNA-protein binding reaction was carried out by incubation of 0.01 μM of DNA probe with 0.5 μM of recombinant proteins in a total volume of 15 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 DTT. After incubating for 20 min at room temperature, the protein-DNA complexes were separated on a 3% native agarose-acrylamide gel. The gel was stained by SYBR Green EMSA stain (Thermo Fisher).

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Chapter 3: Results

3.1 *ERF65*, *ERF70*, *ERF71* and *ERF72* had distinct expression patterns under submergence.

Previous study showed several ERFVIIs, including *OsERF65, OsERF70, OsERF71* and *OsERF72*, in FR13A and IR29 displayed constitutive expression patterns in normal conditions (0hr) and during submergence (Lin et al., 2019). FR13A and IR29 are indica rice that carry *SUB1A-1* and *SUB1A-2* alleles, respectively. TNG67 is a japonica rice that does not have the *SUB1A* allele. Through quantitative RT-PCR (qRT-PCR), the transcript levels of these four genes in 14-day-old TNG67 seedlings under submergence treatment were further examined (Figure 1). The results showed that *ERF65, ERF70, ERF71* and *ERF72* were also constitutively expressed during submergence. The similar expression patterns in different genetic backgrounds suggested the potential roles of *ERF65/70/71/72* to regulate the basal tolerance of rice to submergence stress. The constitutive expression of ERFVIIs would respond at an early stage during submergence, because their proteins would be stabilized quickly at the onset of hypoxia to activate or repress the downstream target genes.

3.2 ERF65, ERF70 and ERF72 could activate the expression of *ERF67*.

Previous studies showed that several ERFVIIs including, *SUB1A*, *SUB1C* and *ERF67*, participated in flooding stress responses. Transient assays in TNG67 rice protoplasts were carried out to determine whether these genes were regulated by ERF65/70/71/72, of which only the regulation of *ERF67* expression displayed reliable results (Figure 2A, the results of *SUB1A* and *SUB1C* were not present). Because

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ERF65/70/71/72 are substrates of N-degron (Chih-Cheng Lin, unpublished data), Nt-MCGG of these 4 ERFs was changed to Nt-MAGG and used in transient assays in order to avoid the oxidation of cysteine and enhance protein stability. SUB1A-1 could directly activate *ERF67* expression and escape from the N-end rule so the wild-type form of SUB1A-1 was used as a positive control (Lin et al., 2019). As was reflected by the ratio of the activity of *Firefly* luciferase to that of *Renilla* luciferase, all four ERFVIIs could activate *ERF67* expression at different levels (Figure 2B). SUB1A-1 activated *ERF67* expression 10-fold compared with the control. ERF65, ERF70 and ERF72 stimulated *ERF67* from 3- to 5-fold while ERF71 activated *ERF67* less than 2-fold.

Since the transcript abundance of *ERF67* was directly up-regulated by SUB1A-1 under submergence, two effectors were co-transformed to determine the influence of ERF65/70/72 on the regulation between SUB1A and the expression of *ERF67*. The results displayed that co-expression of SUB1A-1 respectively with ERF65, ERF70 and ERF72 could enhance 2- to 3-fold activation effects compared to SUB1A-1 only. Co-expression of SUB1A-2 respectively with others had the same effects as SUB1A-1. Interestingly, SUB1A-2 could not induce the expression of *ERF67* through transient expression of SUB1A-2 in protoplasts and then detecting the endogenous transcripts (Wan-Jia Lee, unpublished). The reasons were that transcription mechanisms were more complex and the reporter assays could not determine the epigenetic processes.

Therefore, our lab member Chih-Cheng Lin conducted similar experiments to examine the transcript level of *ERF67* (Figure 3). The results showed that the transcript levels of ERF67 were higher in the SUB1A-1 group than in SUB1A-2 group. The transcript of ERF67 stimulated by SUB1A-1 was 2-fold compared to SUB1A-2. Co-expressions of ERF65, ERF70 or ERF72 with SUB1A-1 could respectively enhance the

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expressions of ERF67 by 6-, 8- and 2-fold. Co-expressions of ERF65/70/72 with SUB1A-2 could enhance ERF67 transcript 2- to 5-fold. It meant that even if *SUB1A-1* and *SUB1A-2* were expressed at the same level, phosphorylation of SUB1A-1 had full functionality to activate the expression of *ERF67*.

3.3 ERF65, ERF70 and ERF72 had similar binding sites on the *ERF67* promoter.

The co-activation effects of SUB1A and ERF65/70/72 might result from synergistic transcription. The promoter containing numerous cis-elements is bound by the same or different transcription factors, and the transcription factors would cooperate and affect the transcription efficiency. Therefore, the potential binding sites of ERF65/70/72 on the *ERF67* promoter were analyzed. The conserved ERF/AP2 domain of ERFVIIs is known to bind the GCC box with a core sequence 5'-GCCGCC-3'. Four GCC boxes in the promoter of *ERF67* were named GCC1 to GCC 4 at the position of -172, -402, -482 and - 1095 upstream the start codon. Continuous deletions for these sites were used to confirm the binding sites of ERF65, ERF70 and ERF72 on the promoter of *ERF67* (Figure 4). The deletion of GCC1 was not performed because the flanking sequence of GCC1 contained the TATA box and its removal might influence the general transcription. For the activation effects of ERF65 or ERF72, the relative luciferase activity decreased with the removal of GCC4 and GCC3. For ERF70, the relative luciferase activity was reduced by removing the region from -1093 to -473 containing GCC3. For SUB1A-1, the relative luciferase activity declined continuously by deleting each GCC box.

The *ERF67* promoter with a GCC mutation (5'-GCCGCC-3' changed to 5'-ATTATT-3') was used to further confirm whether ERF65/70/72 bound the GCC box

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(Figure 5). However, the results were not consistent between promoter deletion and promoter mutation assays. For ERF65, ERF70 and ERF72, the relative luciferase activity was reduced by the mutation of GCC1 but not mutations in GCC3 or GCC4. For SUB1A-1, the relative luciferase activity decreased when respectively mutating GCC1, GCC2 and GCC3. These results indicated ERF65/70/72 might bind GCC1 and SUB1A-1 might bind more than one GCC box on the promoter of *ERF67*. The region from -1200 to -473 in the promoter of ERF67 might contain unknown elements for ERFVIIs binding or indirect regulation.

Although the results of promoter assays were not consistent, the interactions between ERFVIIs and GCC boxes were related to the expression of *ERF67*. Three GCC boxes on *ERF67* promoter simultaneously binding by SUB1A-1 would act synergistic transcription and contribute to the high ability to activate the expression of *ERF67*. These GCC boxes occupied by various ERFVIIs might result in co-activation effects. Therefore, the ERF67 promoter with a GCC mutation was used to confirm the reason for co-activation effects (Figure 5B). Co-activation assays of the GCC4 mutation were not performed because GCC4 was not required for each ERFVII activation. However, the results were not exactly as expected. Co-activation effect of ERF72 with SUB1A-1 was abolished by the mutation of GCC1 and not influenced by the mutation of GCC2 or GCC3, while the effects of ERF65/70 with SUB1A-1 were not influenced by mutation of any GCC box. Even if the co-activation effects were performed by mutating one GCC box, the relative luciferase activity were lower than wild-type of *ERF67* promoter.

3.4 ERF70 and ERF72 could directly bind to the promoter of *ERF67*.

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Because the trans-activation assays using promoter deletion or site-directed mutations could not ensure the binding sites of ERF65/70/72 on the *ERF67* promoter, an electrophoresis mobility shift assay (EMSA) was used to confirm the binding sites (Figure 6). Fluorescein amidite (FAM) labeled DNA fragments containing single GCC box with different flanking sequences from ERF67 promoter were used as probes (Table 3). The results showed that SUB1A-1 could interact with GCC1, GCC2 and GCC3 of the *ERF67* promoter, which was consistent with the results of transient assays. However, neither ERF65, ERF70 nor ERF72 showed interactions with the GCC probes.

The results would come from low binding affinities between the target protein and short fragment probes. If a long fragment was used as a probe, more proteins might bind to it and the cooperative binding would stabilize the DNA-protein complex. Therefore, the long fragment of ERF67 promoter containing both GCC1 and GCC2 was used as a probe to verify whether ERF65/70/72 could directly bind the promoter of ERF67. As the protein-DNA complex was too large to be segregated by 6% acrylamide gel, 3% acrylamide-0.5% agarose gel was used to increase pore size and agarose could strengthen the gel. The results showed that SUB1A-1, ERF70 and ERF72 could directly interact with the *ERF67* promoter but ERF65 had lower or no affinity to this fragment. Using this fragment containing mutations of both GCC boxes as a probe, SUB1A-1, ERF70 and ERF72 could still interact with the *ERF67* promoter, indicating that these proteins still had another binding site on *ERF67* promoter.

3.5 ERFVIIs could regulate the expression of *nsHB*s.

Previous studies showed that up-regulation of *HB1* and *HB2* during submergence in *SUB1A-1*-, *ERF66*- and *ERF67*- overexpression lines through RNA sequence

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analysis (Lin et al., 2019), indicating the expression levels of *HB1* and *HB2* might be regulated by ERFVIIs. Therefore, the transcript levels of *nsHBs* in 14-d-old TNG67 seedlings under submergence treatment were detected through qRT-PCR (Figure 7). The results showed that *HB1* had low abundance and no change during short-term submergence while the expression of *HB2* was induced under 3hr submergence.

Furthermore, transient assays were used to determine whether ERFVIIs could regulate the expressions of *nsHBs* (Figure 8). Based on the up-regulation of *HB1* and *HB2* during submergence in the ERF67-OE lines from RNA-sequence data (Lin et al., 2019), ERF67 was also used to examine the activation effect on the promoters of *nsHBs*. The results showed that ERF65 and ERF70 could activate the promoter of *HB1* six- to nine-fold, while ERF71 and ERF72 had intermediate abilities to stimulate it three- to five-fold. Based on the low abundance of *HB1* during submergence, the regulations of ERFVIIs to *HB1* might be involved in other stresses. ERF65, ERF70 and ERF71 could also induce the promoter of *HB2* twelve-fold, and ERF72 could activate it five-fold. ERF67 and SUB1A-1 activate *HB2* from two- to three-fold. Furthermore, co-expression of ERF71 and ERF67 reduced the expression of *HB2* than ERF71 only while co-expression of ERF70 and ERFF67 showed no difference than ERF70 only. It indicated that ERF71 and ERF67 might compete for the same binding sites, which would fine-tune the expression of *HB2*.

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Chapter 4: Discussions

When the environment changes rapidly, plants need to have sensing and acclimation mechanisms within the second to minute timescale, which is important for fitness and survival of plants (Kollist et al., 2019). During submergence, ERFVIIs with constitutive expressions could quickly accumulate the proteins without *de novo* transcriptions, and then coordinate the downstream genes at early stages. In addition, the expression patterns of *ERF65*, *ERF70*, *ERF71* and *ERF72* were similar in different genetic backgrounds, implying their essential roles in the basal tolerance to submergence.

Previous study showed that the expression level of *ERF67* during submergence was dependent on the activity of SUB1A (Lin et al., 2019). Our result showed ERF65, ERF70 and ERF72 could also induce the expression of *ERF67*, which might be an important regulation in TNG67 (having no SUB1A). However, such inductions were not enough to prolong and achieve the maximal transcription effects of *ERF67* as in tolerant cultivar FR13A. In tolerant cultivars, SUB1A-1 with ERF65/70/72 could induce the expression of *ERF67* efficiently, but it was unknown the necessity of the regulation from ERF65/70/72 to *ERF67*. In addition, from the unpublished data of our lab, the phosphorylation of SUB1A-1 by MPK3 was a key for interacting with remodeler complexes, which would influence the transcriptional effect. Our results showed the ERF65/70/72 with SUB1A could co-activate the promoter of *ERF67* whether SUB1A was phosphorylated or not, but phosphorylated SUB1A-1 was more efficient to induce *ERF67* transcript level.

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Synergistic transcriptional activation results from cooperative DNA-binding and contributes to specific recognition (Veitia, 2003). There are several potential binding sites of the *ERF67* promoter for ERF proteins. SUB1A-1 could bind more than one GCC box on the promoter of *ERF67*, which was related to the ability to regulate the expression of *ERF67*. When the GCC boxes were occupied by different ERF proteins, such as ERF72 and SUB1A-1, they might recruit different factors for transcription or influence promoter structure, improving transcription effects. Although the potential-binding GCC box was removed, the co-activation effects between ERF65/70 and SUB1A-1 were still remained, indicating there were unexpected binding sites which would be discussed in the next paragraph. In addition, it couldn't be ruled out the effects of protein-protein interactions, which could lead to more efficient promoter occupancy (Vashee et al., 1998). Although monomeric ERF domain displayed high DNA binding affinity, ERF proteins are readily able to form homo- and heterodimers with each other (Hao et al., 1998; Cutcliffe et al., 2011).

The interactions between ERF65/70/72 with the *ERF67* promoter were unclear. Although GCC1 in the *ERF67* promoter was required for ERF65/70/72 activation, it showed no interactions between ERF65/70/72 and the GCC boxes of *ERF67* promoter through EMSA assays. Possible reasons for the EMSA results included low binding affinities, indirect bindings or wrong binding sites. The phosphorylation of OsEREBP1(ERF70) by BWMK1 would strongly enhance the binding affinities to synthetic GCC boxes but no significant different to GCC-like boxes from the promoter of *OsRMC* (Cheong et al., 2003; Serra et al., 2013). Whether ERF70 and others could be modified during submergence to influence the binding affinities should be confirmed. Using the labeled-free probes, ERF70 and ERF72 could directly bind the

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ERF67 promoter, and might target other elements independent of the GCC box. Analyzing the regions between GCC1 and GCC2 in the ERF67 promoter through PLACE database, there are two DRE/CRE elements which are also bound by ERF proteins (Lee et al., 2015). As the mutation of GCC1 would decrease the activities of ERF70 and ERF72, it was unknown the effects of *ERFVIIs* target to various elements in the same promoter. Furthermore, the promoter regions between GCC3 and GCC4 were also analyzed based on the results of the promoter deletion assays. The results showed various cis-elements but no ERF-related binding sites were found, indicating there might be unknown elements for ERF protein binding. Another possible reason is that ERF65/70/72 might indirectly regulate *ERF67* expression through interaction with other types of transcription factors.

The regulation of non-symbiotic hemoglobins (*nsHBs*) is dynamic depending on the stages of submergence. Based on the studies in Arabidopsis, ethylene accumulated within minutes during submergence, which could induce the expression of *AHB1* within 1 hours to modulate NO contents and then influence the protein stabilities of ERFVIIs (Loreti et al., 2005; Hartman et al., 2019). In our study, ERFVIIs with constitutive expressions could activate the expression of *HB2* while submergence-induced ERFVIIs had lower abilities, indicating a sequential regulation to prolong the transcript of *HB2*. Co-expression of ERFVIIs containing high and low abilities to activate *HB2* would compete with the binding sites and reduce the expression of *HB2*. Because the expression levels of *ERF67* and *SUB1A* were higher in tolerant plants, it was unknown that such fine-tune regulation was the same in different genetic backgrounds. In *SUB1A*- and *ERF67*-OE lines, the transcript of *nsHBs* under 24-hr submergence were higher than wild-type (TNG67) plants (Lin et al., 2019). Previous studies showed the

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expression of *nsHBs* would limit the formation of ethylene partially through the modulation of NO content, and the production of ethylene would be restricted in a *SUB1A*-dependent manner (Manac'h Little et al., 2005; Fukao et al., 2006; Hebelstrup et al., 2012). It implied the inductions of *nsHBs* dependent on *SUB1A* might partially result in the decrease of ethylene in the submergence tolerant cultivars but the direct correlation should be confirmed.

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Chapter 5: Concluding remarks and future perspectives

As the frequency of severe weather increases, whether plants can overcome diverse pressures is a matter of food. The natural tolerance of rice to flooding has led scientists to study the key regulators. In this study, a set of conserved regulatory pathway provides the basic tolerance of rice to flooding (Figure 8). When the oxygen content decreases during submergence, the constitutive expressions of *ERFVIIs* are ready to coordinate primary response. In tolerant cultivars, the involvement of *SUBA1-1* could enhance the expressions of downstream genes and prolong the survival of plants during submergence. Furthermore, the cooperation and competition among transcription factors could fine-tune gene expression and reflect demand quickly.

In the future, there are some problems left in this study to be solved, including the effects of DNA-binding affinities caused by protein-protein interactions and post-translational modifications. The phenotyping of transgenic lines was not performed as well. In addition, it is also an important question whether this regulatory loop is similar in other crops, contributing to flooding tolerance and why it could not be as tolerant as rice.

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Chapter 6: Tables and figures

Table 1. Primers used for qRT-PCR.

Gene (Accession)	Name	Sequence (5'→3')
OsERF65 (LOC_Os07g42510)	ERF65_qF	GGGATTTCGATGTTGATTGCGATG
	ERF65_qR	AGACCGTTCATGTCGGATTCTTGG
OsERF67 (LOC_Os07g47790)	ERF67_qF	TTCCACCGACTCGTCAGCTTAG
	ERF67_qR	TGCGGGATGAAGTCGGAAATG
OsERF70 (LOC_Os02g54160)	ERF70_qF	TCCTGCAATGAACTCTGCTGCTC
	ERF70_qR	GCAGCCAAATGAGTTGCTTCCC
OsERF71 (LOC_Os06g09390)	ERF71_qF	CGGCTTCGCTAAAGGTGGATTG
	ERF71_qR	CTCTGATTTCCGCAGCCCATTTG
OsERF72 (LOC_Os09g26420)	ERF72_qF	CGAAATGTTCTGGTCAGTGTGGTC
	ERF72_qR	CTTGGCCATACACATTCAACATGG
OsHB1 (LOC_Os03g13140)	HB1_qF	CAACGCTTCCATGCTTCTTG
	HB1_qR	GCAGGTGAGGCCGATTTAT
OsHB2 (LOC_Os03g12510)	HB2_qF	TCCATGATCCTCGCTGA
	HB2_qR	AATCATTTGGACATACACACAC
α-Tubulin (LOC_Os07g38730)	Tubulin_qF	CGCAGTTGCAACCATCAAGACG
	Tubulin_qR	ACTTGAATCCAGTAGGGCACCAG

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Table 2. Primers used for cloning.

Gene	Name	Application	Sequence (5'→3')
OsERF65	ERF65_F	CDS cloning	ATGGCGGGAGGATCCATTCTCG GCGACCTTCACTTGCCGG
	ERF65_R	CDS cloning	ATAAGCTCTGAACTCCATTGGC ATGTCGTCGAAGCTCCAG
OsERF70	ERF70_F	CDS cloning	ATGGCGGGCGGCGCCATCATCC ACCACCTGAAGGGG
	ERF70_R	CDS cloning	ATAGAAATCGCTAACGGGCATG TCATCAAAGCTCCAG
OsERF71	ERF71_F	CDS cloning	ATGGCGGGCGGCGCCATCCTCT CCGACCTCATCCC
	ERF71_R	CDS cloning	GTAGAACTCGGCCGACACGGG CATGTCATCGAAGC
OsERF72	ERF72_F	CDS cloning	ATGGCGGGCGGAGCAATCATCT CCGGGTTCATCCCGCCGTC
	ERF72_R	CDS cloning	GTAGGCACCAGCTGCCATGAGC AGCTCATCAAGGCTCCAG
SUB1A	SUB1A_F	CDS cloning	ATGGTGTGAGGAGAAGTGATCC CCGC
	SUB1A_R	CDS cloning	GGCTTCCCCTGCATATGATATGT TTTG

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OsERF67	ERF67_F	CDS cloning	ATGGCGGGCGGCGCGATCATTTCCGAC
	ERF67_R	CDS cloning	CATCGGCACGGCCGTGTGG
	p67_F	-1200bp promoter cloning	CACATACTACTCCACCACAGT
	p67_R	Promoter cloning reverse	CTTTGCTGCTGCTGCG
	p67_D1	-1073bp promoter cloning	AAAACCATGGTCCGCGCGTAT
	p67_D2	-473bp promoter cloning	CTTCACTAGCCGTAAAACGGGCA
	p67_D3	-393bp promoter cloning	ATCAAGAGTCGGTCAGTCACC
	p67_g1m_F	GCC1 mutation	GTCCGCTCCCCCCGCATTATTACGCTCTTCTATATA
	p67_g1m_R	GCC1 mutation	TATATAGAAGAGCGTAATAATGCGGGGGGAGCGGAC
	p67_g2m_F	GCC2 mutation	CAGAAAGAAAGCAGCATTATTACCGAGCGATCAAGA
	p67_g2m_R	GCC2 mutation	TCTTGATCGCTCGGTAATAATGCTGCTTTCTTTCTG
	p67_g3m_F	GCC3 mutation	TATACTGGAGCAGCGATTATTGTCACCATCTTCACT

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	p67_g3m_R	GCC3 mutation	AGTGAAGATGGTGACAATAATC GCTGCTCCAGTATA
	p67_g4m_F	GCC4 mutation	CAGAGGCAGCCGGCAATTATTC GTGTCCAAAACCAT
	p67_g4m_R	GCC4 mutation	ATGGTTTTGGACACGAATAATT GCCGGCTGCCTCTG
	P67_g12_F	EMSA probe	AAAACCATGGTCCGCGCGTAT
	P67_g12_R	EMSA probe	CTGGTTTATATAGAAGAGCG
OsHB1	pHB1_F	-1500bp promoter cloning	ATAGGTACCTGCCGAGTGCACT CTCCTAC
	pHB1_R	Promoter cloning reverse	TAACCCGGGTGCTTCCTGACAG CTGGTTAATTT
OsHB2	pHB2_F	-1500bp promoter cloning	ATAGGTACCACTGCGACTGGCT GCAC
	pHB2_R	Promoter cloning reverse	TAACCCGGGGGCTGCTTCGATT TGATTCCTCT

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Table 3. Probes used for EMSA.

Probe	Sequence (5'→3')
ERF67 GCC1	CCCCCCGCGCCGCCACGCTCT
ERF67 GCC2	AAAGCAGCGGCGGCACCGAGCG
ERF67 GCC3	GAGCAGCAGCCGCCGTCACCAT
ERF67 GCC4	AGCCGGCAGCCGCCCGTGTCCA

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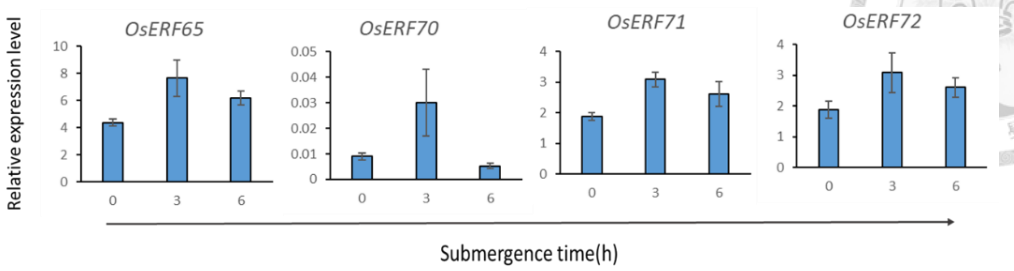

Figure 1. The expression patterns of *ERFVIIs* under submergence.

The shoots were harvested from submergence-treated 14-day-old TNG67 seedlings at different points and the transcript levels of *ERFVIIs* were detected through qRT-PCR. *Tubulin* was used as an internal control for normalization. Relative expression level was determined by ΔCT of the target gene normalized to the internal control. The data represent mean ± SD from three replicates.

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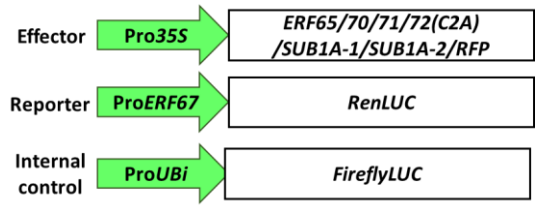
(A)



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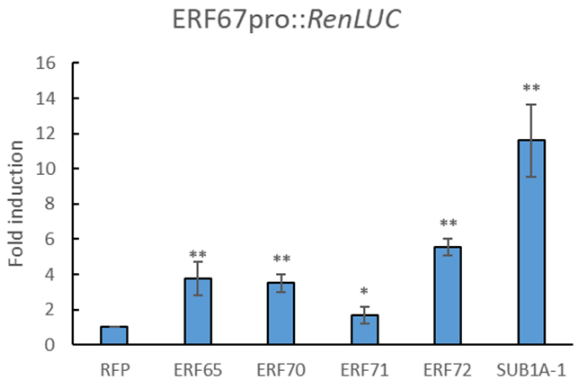

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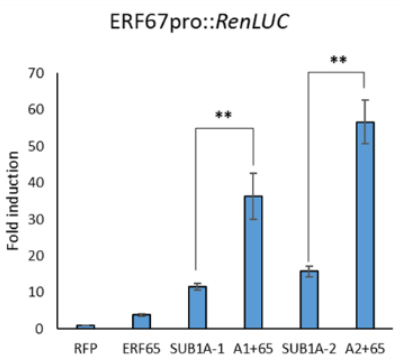

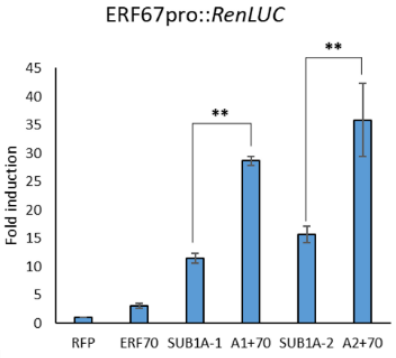

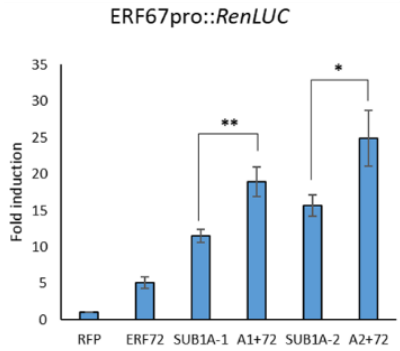

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Figure 2. The activation effects of ERFVIIs on expression of *ERF67*.

(A) Constructs used for *Trans*-activation assays. The coding sequence of *ERF65/70/71/72/SUB1A-1/SUB1A-2* were linked to *35S* promoter for use as effector constructs. C2A indicated the change of Nt-MCGG to Nt-MAGG. The *35SP::RFP* was used as a control. The 1200-bp promoter fragment of *ERF67* was fused to the coding sequence of *Renilla* luciferase to be used as reporter constructs. The *UbiP::Firefly Luciferase* was used as the internal control. The activation effects of single ERFVIIs (B) and co-activation of *SUB1A* with ERF65/70/72 (C) to *ERF67* promoter were performed in TNG67 protoplasts. The fold induction represented the relative luciferase activity (calculated as the ratio of *RLuc* activity/*FLuc* activity) between the effector genes and control. The data represent mean ± SD from at least four replicates. (*$P < 0.05$ and **$P < 0.01$ indicated significant differences by Student's t test)

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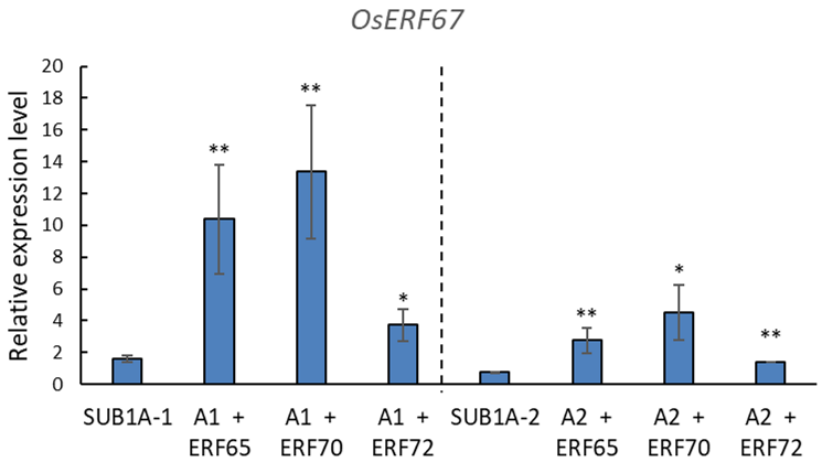

Figure 3. The co-activation effects of SUB1A with ERF65/70/72 to *ERF67* transcription.

After transient expression of SUB1A-1, SUB1A-2, ERF65/70/72 and SUB1A-1 or ERF65/70/72 and SUB1A-2 in TNG67 protoplasts, the transcript level of ERF67 were detected through qRT-PCR. *Tubulin* was used as an internal control for normalization. Relative expression level was determined by ΔCT of the target gene normalized to the internal control. The data represent mean ± SD from three replicates. (*P < 0.05 and **P < 0.01 indicated significant differences by Student's t test). The experiment was conducted by our lab member Chih-Cheng Lin.

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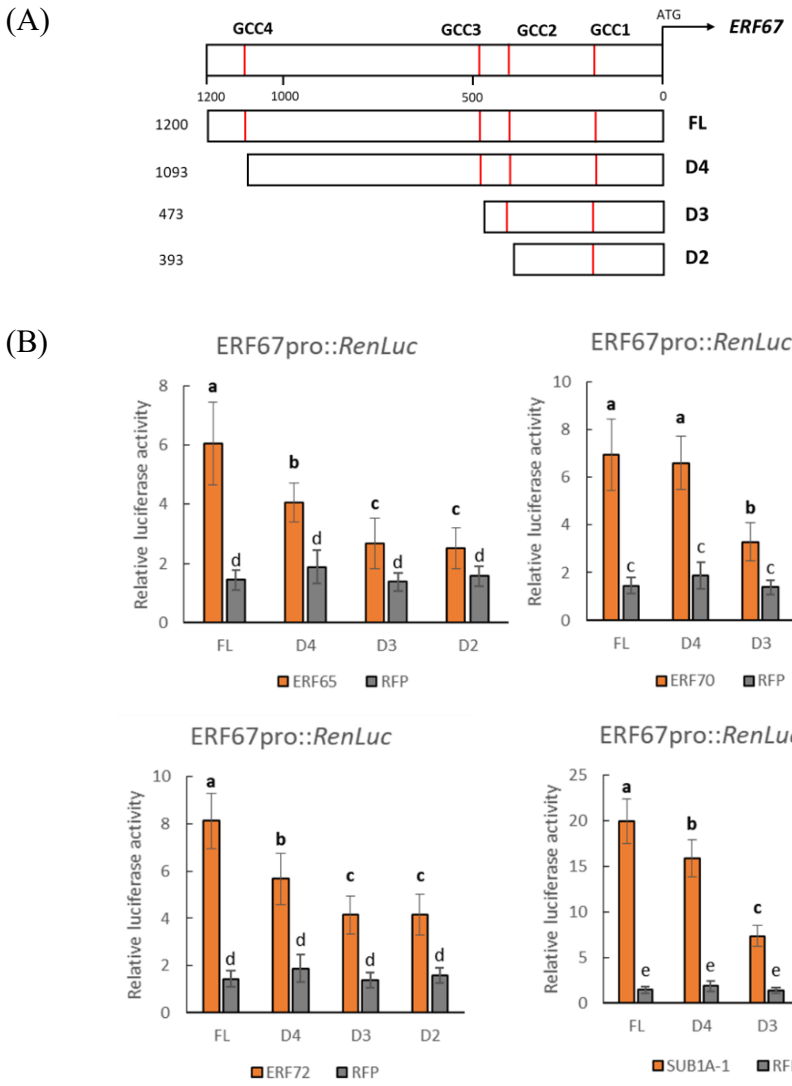

Figure 4. Deletion analysis of the *ERF67* promoter.

(A) The simple diagram represents the position of the GCC box in the promoter of *ERF67*, and the 5' deletion strategy from -1200 to -393 position. (B)The activation effects of ERFVIIs to the deletion promoter of *ERF67* were performed in TNG67 protoplasts. The relative luciferase activity was calculated as the ratio of *RLuc* activity/*FLuc* activity. The data represented mean ± SD from at least four replicates. Different letters indicated significant differences by one-way ANOVA ($P < 0.05$).

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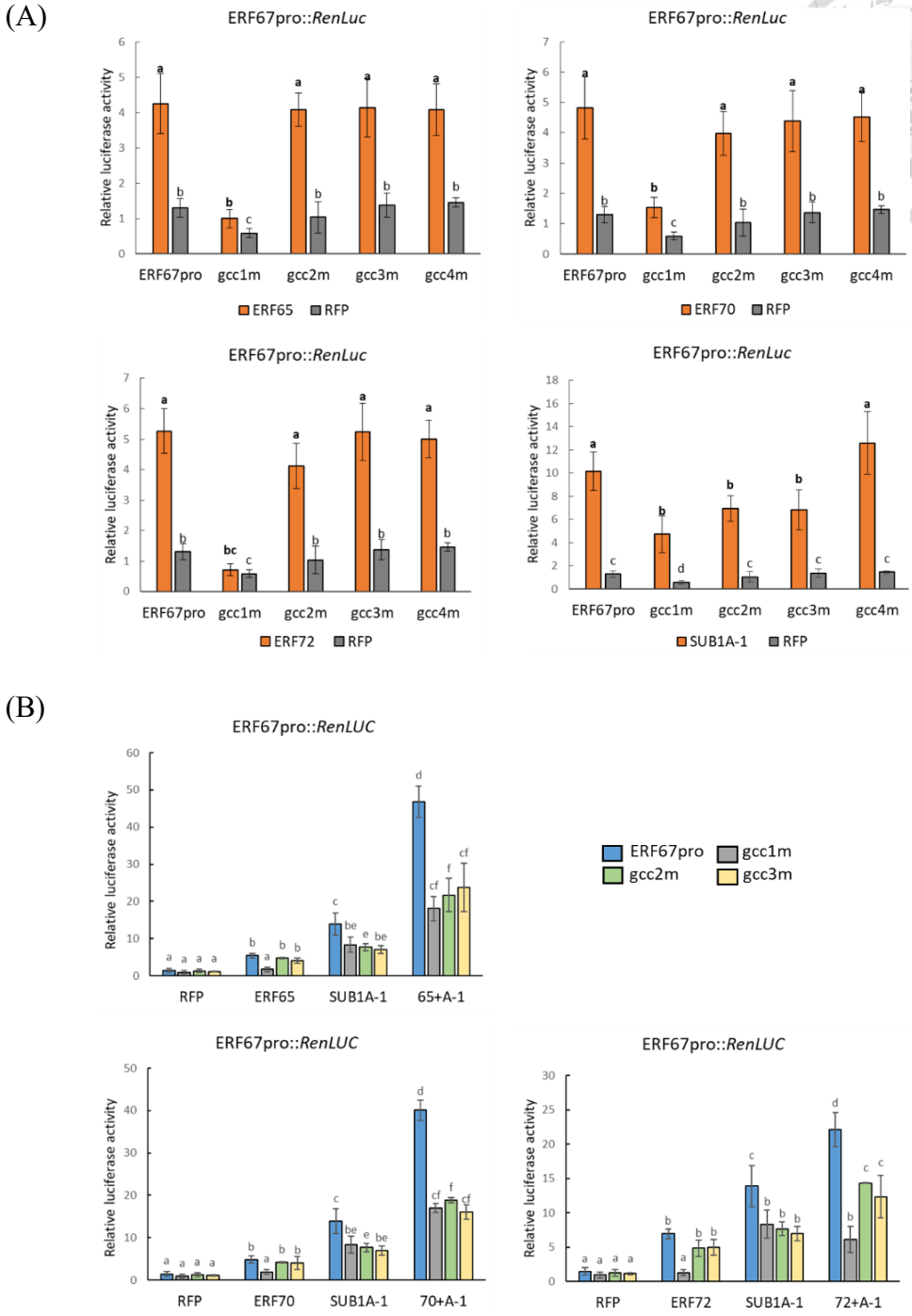

Figure 5. Effects of GCC box mutation on *ERF67* expression.

The activation effects of single ERFVIIs (A) and co-activation effects of SUB1A-1 respectively with others (B) to the *ERF67* promoter containing single GCC box mutation were performed in TNG67 protoplasts. gcc1m, gcc2m, gcc3m and gcc4m

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indicated the respective mutation of GCC1, GCC2, GCC3 and GCC4 on the *ERF67* promoter. The relative luciferase activity was calculated as the ratio of *RLuc* activity/*FLuc* activity. The data represented mean ± SD from at least four replicates. Different letters indicated significant differences by one-way ANOVA ($P < 0.05$).

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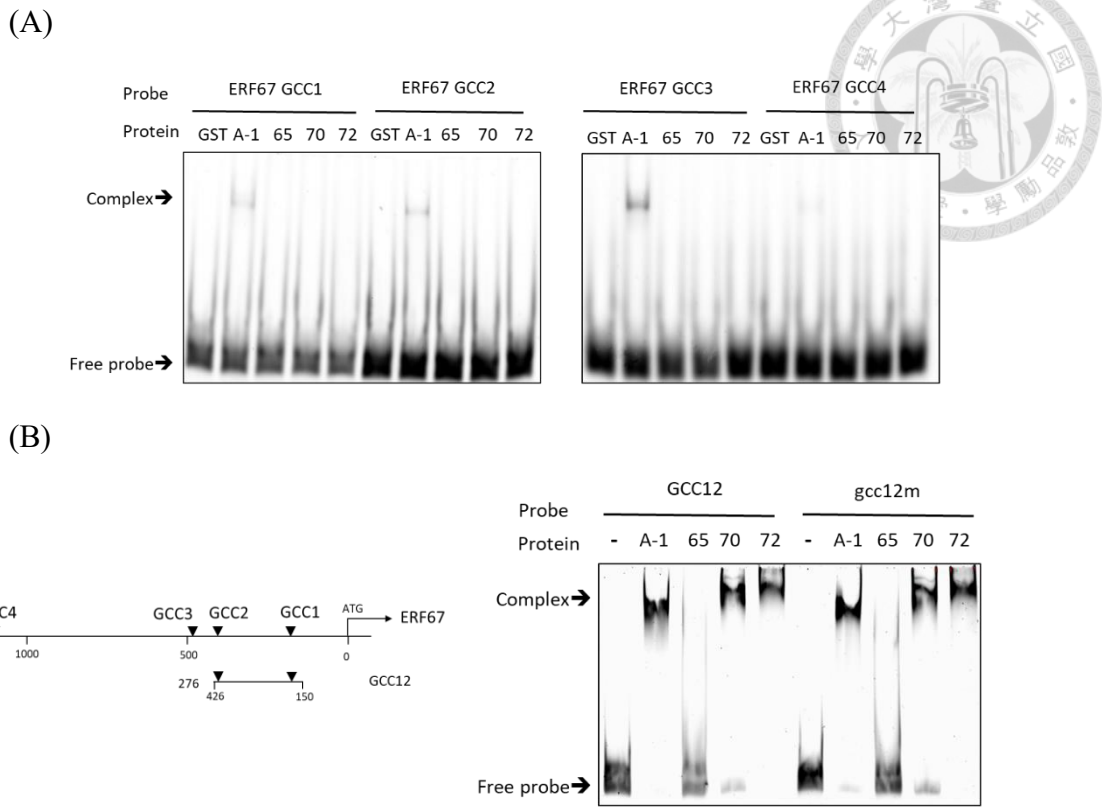

Figure 6. The interaction between ERFVIIs and the promoter of *ERF67 in vitro*. The recombinant proteins of SUB1A-1, ERF65, ERF70 and ERF72 were incubated with FAM-labeled DNA (A) or labeled-free DNA (B) and separated by native-gel. (A) Each GCC from the promoter of *ERF67* contained different flanking sequences. The probe sequences were listed at Table 3. (B) The left diagram represents the 276-bp probe containing GCC1 and GCC2 at the *ERF67* promoter from -33 to -309. The probe of gcc12m indicated both of the GCC boxes (5'-GCCGCC-3') was mutated to 5'-ATTATT-3'.

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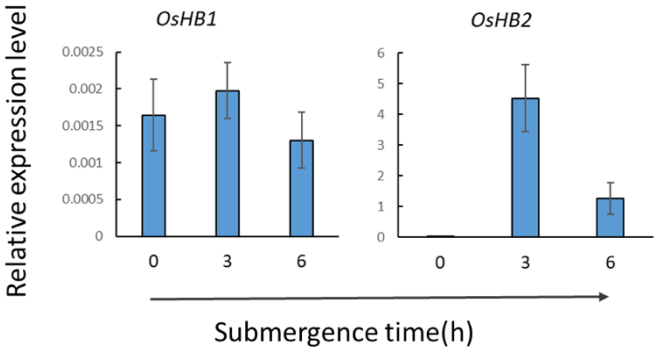

Figure 7. The expression patterns of non-symbiotic hemoglobins (*nsHBs*) under submergence.

The shoots were harvested from submergence-treated 14-day-old TNG67 seedlings at different points and the transcript levels of *nsHBs* were detected through qRT-PCR. *Tubulin* was used as an internal control for normalization. Relative expression level is determined by ΔCT of the target gene normalized to the internal control. The data represent mean ± SD from three replicates.

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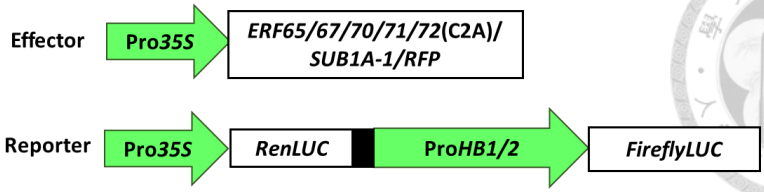
(A)



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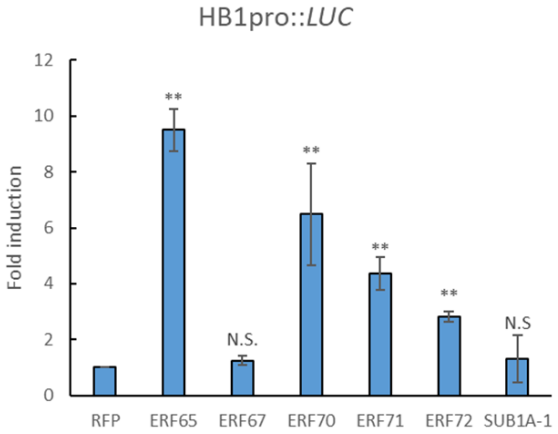

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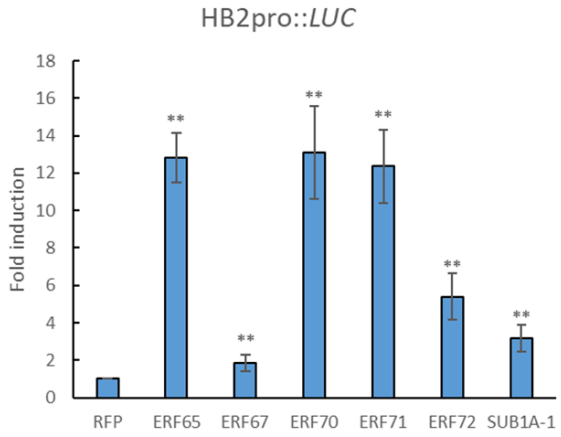

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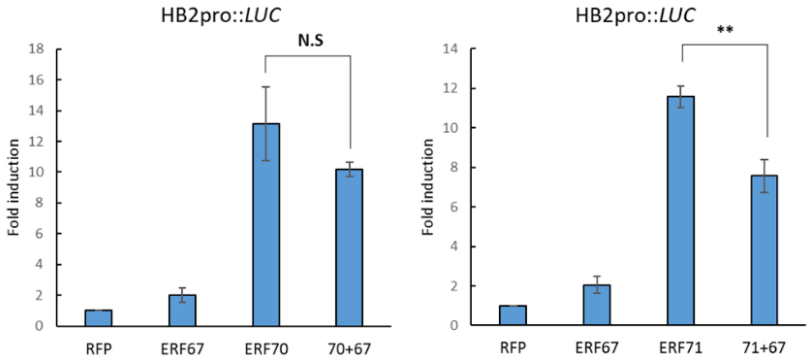

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Figure 8. The activation effects of ERFVIIs on expression of *nsHBs*.

(A) Constructs used for *Trans*-activation assay. The coding sequence of *ERF65/67/70/71/72/SUB1A-1* were linked to *35S* promoter for use as effector constructs. The *35SP::RFP* was used as the control. The 1500-bp promoter fragment of *HB1* and *HB2* was fused to the coding sequence of *Firefly* luciferase to be used as reporter constructs. The *35SP::Renilla Luciferase* on reporter construct was used as an internal control. The activation effects of single ERFVIIs to the promoter of *HB1* (B) and *HB2* (C) were performed in TNG67 protoplasts. (D) Co-expression of ERF67 with ERF70 or ERF71 affected the activation of the promoter of *HB2*. The fold induction represented the relative luciferase activity (calculated as the ratio of *FLuc* activity/*RLuc* activity) between the effector genes and control. The data represent mean ± SD from four replicates. (*P < 0.05 and **P < 0.01 indicate significant differences by Student's t test)

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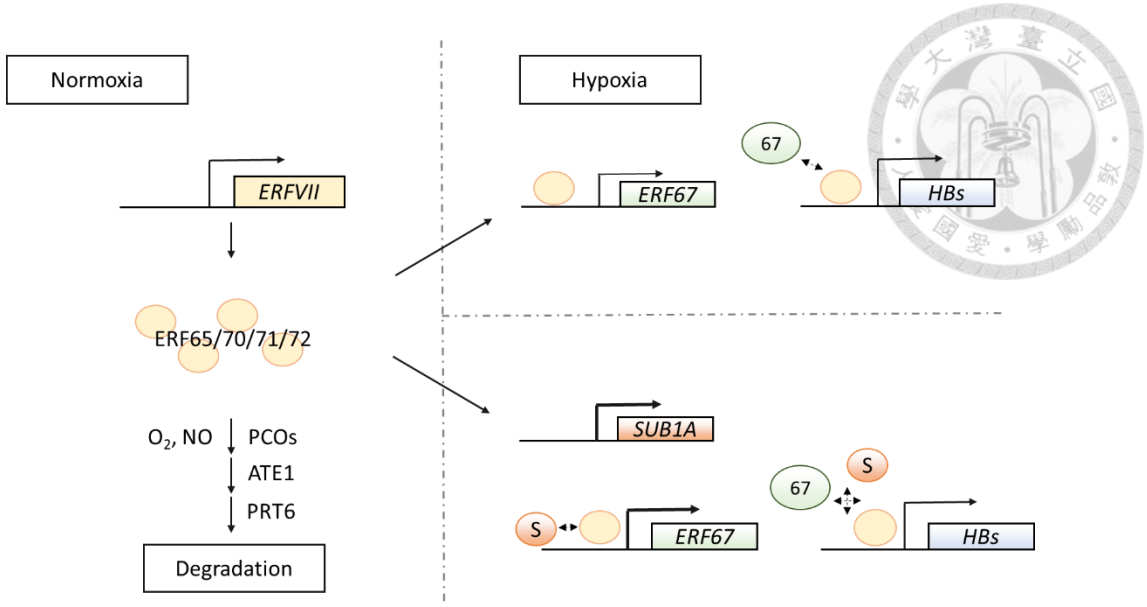

Figure 9. The regulatory loop of *ERFVIIs* in the study.

In normal conditions (normoxia), *ERF65/70/71/72* were constitutively expressed and their proteins were degraded through N-end rule. During submergence, their proteins were stable quickly, and regulated the expressions of *ERF67* and other hypoxia-response genes, including *nsHBs*. In submergence-tolerant cultivars, the involvement of SUB1A could induce the expression of *ERF67*. The coordination and competition of ERFVIIs could fine-tune the expressions of *ERF67* and *nsHBs*. The orange, red and green balls respectively indicated the proteins of ERF65/70/71/72, SUB1A and ERF67.

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