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Functional Roles of Group VII ERFs Through Transient Transformation of Protoplasts in
Arabidopsis and Rice

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Master of Science

in

Biochemistry and Molecular Biology

by

Elaine Yee-Ling Yeung

August 2013

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Dr. Julia Bailey-Serres, Chairperson

Dr. Thomas Eulgem

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2013

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ABSTRACT OF THE THESIS

Functional Roles of Group VII ERFs Through Transient Transformation of Protoplasts in
Arabidopsis and Rice

by

Elaine Yee-Ling Yeung

Master of Science, Graduate Program in Biochemistry and Molecular Biology
University of California, Riverside, August 2013
Dr. Julia Bailey-Serres, Chairperson

Environmental stresses involving flooding and the accompanying cellular low oxygen (hypoxia) constitute a major limitation to agricultural production worldwide. These stresses hinder the plant's ability to maintain energy production and growth. Yet, previous studies have shown that certain genes of group VII ethylene responsive factor (ERF) family of transcription factors play a functional role in the survival of submergence and low oxygen stresses in *Oryza sativa* (rice) and *Arabidopsis thaliana*. This study utilized a transient gene transfection assay of protoplasts to characterize gene networks regulated by the group VII ERFs by measuring the transcriptional activation of various promoters. Experiments revealed that orthologous genes are regulated by the group VII ERFs of both species. In rice, an ERF-type transcription factor, *SUB1A*, confers submergence tolerance by regulating carbohydrate metabolism, elongation growth, and hormonal responses. *SUB1A* is linked to the related genes *SUB1B* and *SUB1C*. Here, two alleles of *SUB1A* (*SUB1A-1*, *SUB1A-2*) as well as one allele each of

the other two genes (*SUB1B-1* and *SUB1C-1*) were shown to be transcriptional activators, and the nuclear localization of *SUB1A-1* and *SUB1C-1* was demonstrated. In *Arabidopsis*, core hypoxia responsive genes are governed by the N-end rule pathway of targeted proteolysis, in which proteins of the group VII ERFs (i.e., RAP2.12 and HRE2) are regulated post-translationally under hypoxic conditions. Transactivation experiments demonstrated that the trihelix protein HRA1 negatively regulates the transcriptional activation ability of RAP2.12 in the core hypoxia response. By contrast, HRE2 was not targeted by HRA1 downregulation, but was instead upregulated by RAP2.12 during the stress. Experiments involving truncations of HRA1 reveal that the coil-coil domain is functionally important for HRA1 interaction with group VII ERF complexes. These results obtained with a rapid protoplast assay provide a foundation for future investigations into the low oxygen response gene network regulated by group VII ERF transcription factors binding to *cis*-regulatory elements, and modulated by the trihelix protein HRA1.

TABLE OF CONTENTS

Abstract.....	v
Chapter 1. Introduction	1
Chapter 2. Materials and Methods.....	25
2.1 Plant materials and growth conditions	25
2.2 Construction of plasmids	25
2.3 Protoplast isolation.....	33
2.4 Protoplast transfection	34
2.5 Dual-luciferase assay	34
2.6 Fluorescence imaging of GFP and nuclear DNA	35
Chapter 3. Results	36
3.1 Dual luciferase system as a method of detection	36
3.2 Functional study of <i>35S:C2A-HRE2</i> in <i>Arabidopsis</i> using <i>HRA1</i> and <i>PDC1</i> promoters	39
3.3 RAP2.12 activates the <i>Arabidopsis</i> promoter but its action is repressed by <i>HRA1</i>	45
3.4 Interaction of truncated <i>HRA1</i> with <i>HRE2</i>	50
3.5 Analysis of <i>SUB1A-1</i> , <i>SUB1A-2</i> , <i>SUB1C-1</i> , and <i>SUB1C-2</i> as transcriptional activators in rice and <i>Arabidopsis</i> protoplast.....	54
3.6 Transfection of <i>Arabidopsis</i> protoplasts to evaluate the capability of <i>SUB1A-1</i> , <i>SUB1A-2</i> , <i>SUB1C-1</i> , and <i>SUB1C-2</i>	

as transcriptional activators.....	60
3.7 Analysis of SAB18 as a negative regulator of <i>SUB1A</i>	62
3.8 Localization of GFP tagged SUB1A-1 and SUB1C-1.....	62
3.9 Measure of protoplast viability using <i>35S::RrLUC</i>	72
Chapter 4. Discussion	74
References	83

LIST OF FIGURES

Figure 1. Hypothesized model of the overlap in regulation by HRA1 and RAP2.12 under low oxygen stress based on current knowledge in <i>Arabidopsis</i>	10
Figure 2. Schematic illustration of the effector and reporter constructs used in the protoplast transient expression studies	38
Figure 3. HRE2 transactivates the <i>HRA1</i> promoter	41
Figure 4. HRE2 activates the <i>Arabidopsis PDC1</i> promoter in <i>Arabidopsis</i> protoplasts.....	42
Figure 5. HRE2 activates the <i>Arabidopsis HRA1</i> promoter but its action is repressed by HRA1	43
Figure 6. HRA1 activates the <i>Arabidopsis PDC1</i> promoter and promotes <i>C2A-HRE2</i> activation of the <i>PDC1</i> promoter in <i>Arabidopsis</i> protoplasts.....	44
Figure 7. Positive regulation of the <i>HRA1</i> promoter by <i>35S:Δ13RAP2.12</i> is reversed by co-transfection with <i>35S:HRA1</i> in <i>Arabidopsis</i> protoplasts.....	47
Figure 8. Positive regulation of the <i>PDC1</i> promoter by <i>35S:Δ13RAP2.12</i> is inhibited by <i>35S:HRA1</i> co-transfection in <i>Arabidopsis</i> protoplasts.....	48
Figure 9. Co-transfection of <i>35S:Δ13RAP2.12</i> with <i>35S:C2A:HRE2</i> and <i>35S:HRA1</i> in <i>Arabidopsis</i> protoplasts.....	49
Figure 10. Illustration of the N- or C- terminal amino acid deletions in the	

truncated HRA1 construct, and definition of the nomenclature	52
Figure 11. Transient expression of truncated <i>HRA1</i> in <i>Arabidopsis</i> protoplasts	53
Figure 12. <i>PromOsCIPK15:PpLUC</i> is activated by <i>35S:SUB1A-1</i> in transiently expressed rice protoplasts	57
Figure 13. Transient expression of <i>35S:HRA1</i> promoting <i>35S:SUB1A-1</i> activation of <i>promOsPDC1:PpLUC</i> in rice protoplasts	59
Figure 14. <i>35S:SUB1A-1</i> , <i>35S:SUB1A-2</i> , <i>35S:SUB1B-1</i> , and <i>35S:SUB1C-1</i> activates <i>promAthPDC1:PpLUC</i> when transiently expressed in <i>Arabidopsis</i> protoplasts	61
Figure 15. Subcellular localization of N- and C- terminal GFP fusion of <i>SUB1A-1</i> and <i>SUB1C-1</i> transiently expressed in <i>N. benthamiana</i> and <i>Arabidopsis</i> protoplasts	65
Figure 16. Subcellular localization of N- and C- terminal GFP fusion of <i>SUB1A-1</i> and <i>SUB1C-1</i> transiently expressed in rice protoplasts	70
Figure 17. Measure of <i>Arabidopsis</i> protoplast survivability using <i>35S:RrLUC</i> and overexpressed <i>HRA1</i> , <i>C2A-HRE2</i> , <i>Δ13RAP2.12</i> constructs	73
Figure 18. Proposed model describing the overlapping regulation of HRA1 on RAP2.12 and HRE2 under low oxygen stress in <i>Arabidopsis</i>	78

LIST OF TABLES

Table 1. Comparison of methods for examining direct regulation by a transcription factor	21
Table 2. Sequences of forward (F) and reverse (R) primers used in this work for constructing and sequencing plasmids	28
Table 3. List of all plasmids used in this transient expression study.....	29

Chapter 1

INTRODUCTION

The world faces a significant crisis today as rapid population growth continues to elevate the demand for production of staple cereal crops, including maize (*Zea mays*), wheat (*Triticum* spp.), and rice (*Oryza sativa*). This situation is exacerbated by global climate change that increases the frequency and duration of weather extremes causing floods, drought, and temperature extremities, all of which threaten the stable production of primary crops. Flooding has an especially detrimental effect on crop production, as 35% of the world's farmland for rice production is prone to flood (Bailey-Serres et al., 2012b). The occurrences of floods have increased in the last six decades, with an exponential increase in the past decade in Asia and Africa, where food insecurity poses an even more severe crisis (Bailey-Serres et al., 2012a). Consequently, there is need for improvement of these crops in regards to their adaptations and survival from the stress of flooding stresses. This thesis focuses on transcription factors associated with responses to low oxygen stress in *Arabidopsis* and submergence in rice.

Transcriptional response to low oxygen

Oxygen deficiency is a detrimental consequence of flooding, either through flooding of the root system (waterlogging), or partial to complete submergence of the plant's aerial tissue under water (Bailey-Serres et al., 2012a, 2012b). In normal well-aerated cultivation or culture conditions (i.e., normoxia), there is typically 20.6% oxygen at 1 atm and 20°C.

Under a low oxygen environment (hypoxia), the level of oxygen falls below 20.6%, but by definition is above complete oxygen depletion (anoxia) (Bailey-Serres et al., 2012b). The K_m for mitochondrial cytochrome *c* oxidase is 140 nM (~0.013%) O₂ (Bailey-Serres and Voeselek, 2008), enabling ATP production by oxidative phosphorylation to continue until oxygen is nearly absent. Nonetheless, external oxygen deprivation by replacement of air with gas containing low or no oxygen, or flooding has consequences on anaerobic metabolism. The consequences include decreased mitochondrial metabolism resulting in reduced ATP production (Bailey-Serres and Voeselek, 2008; Igamberdiev and Hill, 2009) due to a shift to anaerobic metabolism. This leads to elevation in metabolites including ethanol and alanine (van Dongen et al., 2004; Narsai et al., 2009). Although the impact of low oxygen stress on metabolism generally hinders plant developmental processes, it provides a means for short term survival of the stress.

Hypoxia stress is accompanied by transcriptional and posttranscriptional regulation of gene expression. Dynamics in gene regulation have been reported in numerous plant species. There is a network of approximately one-hundred hypoxia responsive genes, based on a cross species comparison of low-oxygen and flooding induced genes (Mustroph et al., 2010). In *Arabidopsis* these include the genes *PYRUVATE DECARBOXYLASE 1* (*PDC1*, At4g33070), *ALCOHOL DEHYDROGENASE* (*ADH*, At1g77120), non-symbiotic haemoglobin (*HBI*, At2g16060), *HYPOXIA RESPONSE ATTENUATOR 1* (*HRA1*, At3g10040), *HYPOXIA RESPONSIVE ERFs* (*HRE1*, At1g72360, and *HRE2*, At2g47520) (Licausi et al., 2010b, 2011; Lee et al., 2012; Mustroph et al., 2009, 2010). There is evidence through mutant studies that all of these

genes are significant in the regulation of central carbon metabolism and energy conservation. Under hypoxia, there is also post-transcriptional gene regulation. This has been demonstrated as the repression of translation of many cellular mRNAs, a metabolically expensive process, thereby limiting the consumption of ATP during the stress (Branco-Price et al., 2008). Forty-nine “core” hypoxia responsive genes were recognized that circumvent this translational repression across cells and tissues of both the root and shoot of *Arabidopsis* seedlings (Mustroph et al., 2009). The mechanisms that regulate these genes at the transcriptional levels are the topic of this thesis.

Ethylene responsive factor gene family

It has been proposed that in both rice and *Arabidopsis thaliana*, there is a correlation between the functionality of the group VII ERFs critical for survival of low oxygen responses and submergence. The group VII ERFs are part of the ethylene responsive factors (ERF) family of transcription factors that play a significant roles in transcriptional regulation of plant growth, development, and responses to abiotic stress (Nakano et al., 2006; Jung et al., 2010; Yang et al., 2011). Transcription factors are proteins that bind specific DNA sequences, generally termed *cis*-regulatory elements, in a manner that regulates the activity of RNA polymerase II. The *cis*-elements are often located upstream of the transcription start site, but also can be located elsewhere. Transcription factors generally activate and/or repress transcription in a spatial, temporal, or conditional manner, and often constitute a network in which one protein regulates the expression of multiple genes (Saleh and Pagés, 2003). The ERF family is part of the AP2/ERF

superfamily, and the founding members of this superfamily are *APETALA2* (*AP2*), involved in floral patterning, and the *RELATED TO ABI3/VP1* (*RAV*) proteins (Jofuku et al., 1994; Nakano et al., 2006; Licausi et al., 2010a, 2010b). The AP2/ERF superfamily shares a common ERF DNA binding domain of approximately 60 to 70 amino acids (Nakano et al., 2006). The AP2/ERF domain was originally identified in four ethylene-responsive element-binding proteins (*EREBP*) of tobacco, and binds to a GCC box (5'-TAAGAGCCGCC-3'), an 11 bp sequence involved in gene transcription in response to ethylene (Ohme-Takagi and Shinshi, 1995). Genes in the AP2 family have two copies of the AP2/ERF DNA binding domain, while other family members, such as the group VII ERFs, only have a single copy. The RAV family of proteins has two different DNA-binding domains: AP2/ERF and B3 (Sakuma et al., 2002; Saleh and Pagés, 2003; Nakano et al., 2006). Each subfamily of the AP2/ERF proteins is characterized by conservation within the ERF domain, among other features. Proteins in the same subgroup also have conserved motifs outside of the AP2/ERF domain, including those responsible for transcriptional control and nuclear localization (Nakano et al., 2006). The genes of the over 100-member ERF family are further categorized into groups based on their conserved functional motifs. The ERF families were divided into twelve groups in *Arabidopsis thaliana* and fifteen groups in rice. Eleven of these groups are conserved in both plant species (Nakano et al., 2006). The group VII ERFs are characterized by strong conservation in their ERF domain as well as an N-terminal motif MCGGAI(I/L) (Tournier et al., 2003).

N-end rule pathway of targeted proteolysis acts in low oxygen sensing and transcriptional regulation

The transcriptional regulation of many core hypoxia responsive genes in *Arabidopsis* is governed by the N-end rule pathway of targeted proteolysis (Gibbs et al., 2011; Licausi et al., 2011; Sasidharan and Mustroph, 2011). This pathway constitutes a proteolytic cleavage system in which the half-life of proteins is dependent upon their N-terminal residues (Varshavsky, 2011). Specific ubiquitin ligases recognize certain N-terminal primary destabilizing residues, such as basic residues (i.e., Arg, Lys and His) or bulky hydrophobic residues (i.e., Phe, Trp, Tyr, Leu and Ile) (Graciet et al., 2010; Graciet and Wellmer, 2010; Tasaki et al., 2012).

As mentioned, group VII ERFs have a conserved amino-terminal sequence, MCGGAI(I/L). The *Arabidopsis* genome encodes five group VII ERFs: *RELATED TO AP2 12* (*RAP2.12*, At1g53910), *RELATED TO AP2 2* (*RAP2.2*, At3g14230), *RELATED TO AP2 3/ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEIN* (*RAP2.3/EBP*, At3g14230), *HYPOXIA REponsive1* (*HRE1*) and *2* (*HRE2*). The first two residues of the N-terminal motif in group VII ERFs, Met and Cys, are significant for regulation by the N-end rule pathway. The second amino acid of the N-terminal motif, Cys, has been shown to be a destabilizing residue of all five group VII ERFs in an *in vitro* assay and under oxygen replete conditions for HRE1 and HRE2 in transgenic *Arabidopsis* (Gibbs et al., 2011). It was proposed that the N-end rule pathway is initiated when the N-terminal methionine is cleaved from a group VII ERF protein by methionine aminopeptidase (MetAP). The N-terminal Cys is likely to be subsequently oxidized, making it a substrate

for an arginine transferase (*ATE1/2*, At5g05700/At3g11240), which conjugates an Arg residue to the N-terminus of the protein. The Arg-Cys_{ox}-N terminus allows recognition by an E3 ubiquitin ligase, *PROTEOLYSIS 6* (*PRT6*, At5g02310), which mediates ubiquitination of the protein, thus tagging it for 40S proteasome mediated degradation (Lee et al., 2005; Graciet and Wellmer, 2010). In the case of the group VII ERFs HRE1 and HRE2, *ate1 ate2* or *prt6* loss-of-function mutants stabilize the protein under normoxic conditions (Gibbs et al., 2011). Mutation of the second residue of these proteins from Cys to Ala also renders them stable.

RAP2.12 stability was also shown to be regulated by the N-end rule at the cellular level. The gene encoding this protein is constitutively expressed and its mRNA is abundantly translated (Mustroph et al., 2009). The protein localized to the plasma membrane under normoxia and re-localized to the nucleus under hypoxia (Licausi et al., 2011). However, when the first 13 conserved N-terminal amino acids were removed (*35S::Δ13RAP2.12*), *Δ13RAP2.12* localized to both the plasma membrane and the nucleus under aerobic conditions. The mutation of the second amino acid to Ala was also sufficient to render the protein stable under oxygen replete conditions. Importantly, these complementary studies showed that both the native and N-terminally mutated forms of these group VII ERFs were stabilized under low oxygen conditions (Gibbs et al., 2011; Licausi et al., 2011). These results indicate that the accumulation of group VII ERFs is regulated post-translationally in a manner mediated by oxygen availability.

RAP2.12 has also been shown to bind to a *cis*-acting ATCTA promoter element of the *ADH1* promoter (Papdi et al., 2008) and the *HBI* promoter (Licausi et al., 2011).

The ATCTA motif was thus proposed as a hypoxia responsive element (Welsch et al., 2007; Licausi et al., 2011). The ATCTA motif is also present in the promoter regions of *PDC1* and *HRA1*. However, the recognition site for the motif is not in the *HRE2* 5' flanking sequence, suggesting that RAP2.12 may not directly bind the HRE2 promoter or the ATCTA motif may not be a specific factor required for the hypoxic response.

Role of group VII ERFs in gene transcription in *Arabidopsis*

Transcription factors of the *Arabidopsis* group VII ERFs have been shown to enhance plant responses to hypoxia or anoxia (Hinz et al., 2010; Mustroph et al., 2010; Licausi et al., 2010b, 2011; Gibbs et al., 2011; Lee et al., 2011; Yang et al., 2011; Bailey-Serres et al., 2012a, 2012b; Banti et al., 2013). Microarray analysis identified approximately 50 core hypoxia-responsive genes in *Arabidopsis* that are expressed in response to oxygen deprivation as well as submergence (Mustroph et al., 2009, 2010; Lee et al., 2012). This co-regulation network includes genes involved in anaerobic metabolism such as *ADH1*, *PDC1*, *SUCROSE SYNTHASE1* (*SUS1*, At5g20830), *SUCROSE SYNTHASE4* (*SUS4*, At3g43190), and *HBI* (Mustroph et al., 2009, 2010; Lee et al., 2011). As hypoxia responsive-gene transcripts are generally reduced upon re-oxygenation (Branco-Price et al., 2008), these genes must have both positive and negative regulators involved in the response to the environmental stimuli.

It has been shown that survival of the plant under hypoxic stress depends on a “balancing act” (Fukao and Bailey-Serres, 2004). In one instance of the need for balance, a negative-feedback regulation loop mediated by the activation and inactivation of a Rho

G-protein of plant (Rop) counteracts against the severe stress response that results in negative effects, such as excessive carbohydrate consumption and production of reactive oxygen species (Baxter-Burrell et al., 2002). Under low oxygen and submergence conditions in *Arabidopsis*, specific group VII ERF family members could play a key role in the positive and negative regulation of the plant's survival response. This is supported by findings that manipulation of group VII family member expression affects transcript accumulation of a number of hypoxia responsive genes, particularly under low oxygen stress when the proteins accumulate (Lee et al., 2011; Licausi et al., 2011; Gibbs et al., 2012; Lee, 2012).

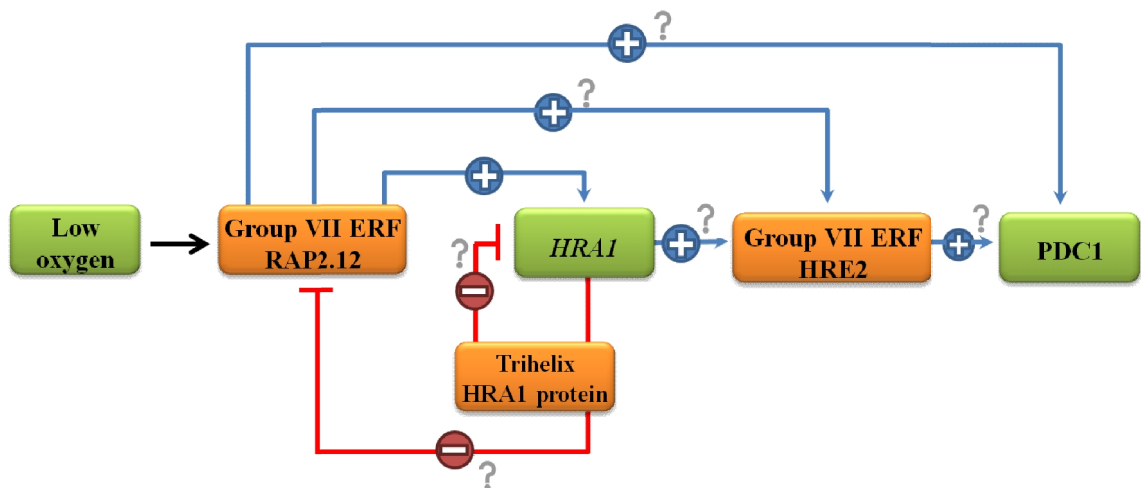
A second transcription factor is likely to play a role in regulation of the core hypoxia-responsive genes. HRA1, a ~48.9 kDa protein that contains a trihelix domain and functions as a transcriptional activator in yeast, was first recognized as a core hypoxia-responsive gene encoding a protein of no known biological function. The ectopic overexpression of HRA1 negatively regulates the transcript accumulation of most core hypoxia responsive genes, including many that are positively regulated by RAP2.12, significantly limiting their accumulation during the stress (Lee, 2012). Overexpression of *HRA1* suppresses induction of *ADH* activity in seedlings under hypoxic conditions. Meanwhile, RAP2.12 overexpression activated the promoter of *ADH1*, though it is unknown whether this enhancement is comparable with hypoxic stress (Papdi et al., 2008). It is also yet to be determined whether HRA1 acts as negative regulator of group VII ERFs such as RAP2.12 or independently. The involvement of both group VII ERFs and HRA1 in regulation of *ADH1* transcript accumulation led us to hypothesize that these

proteins may co-regulate a gene network under low oxygen stress.

The group VII ERFs HRE1 and HRE2 may be redundant, overlapping, or unique regulators of hypoxia-responsive genes relative to RAP2.12 (Figure 1). All three ERFs are regulated by the N-end rule pathway, but it is not clear if they upregulate the same or specific anaerobic genes under low oxygen in *Arabidopsis*. In seedlings, *HRE1* is highly induced by hypoxia in roots but not shoots, whereas *HRE2* is more ubiquitously upregulated (Mustroph et al., 2009). *In vivo* studies of seedlings confirmed elevation of C-terminally HA-tagged HRE2 under hypoxic conditions (Gibbs et al., 2011). By contrast, HRE1-HA was not above the threshold for detection. An early study indicated that induction by anoxia of *HRE1* mRNA requires protein synthesis, whereas that of *HRE2* does not (Licausi et al., 2010b), suggesting that the regulation of transcripts encoding these ERFs may not be the same. *HRE2* was proposed to be regulated by RAP2.12, leading to the suggestion that RAP2.12 initiates gene transcriptional activation in response to low oxygen survivability. But the other group VII ERFs may be redundant to RAP2.12, since the low oxygen survival of single mutants, *rap2.12* or *rap2.3*, was not compromised (Hinz et al., 2010; Yang et al., 2011). HRE1 and HRE2 are likely to be important in the hypoxic response, although a double *hre1 hre2* but not a single *hre1* or *hre2* mutant displayed increased low oxygen sensitivity even though the mutants showed elevated ADH enzyme activity and ethanol production during hypoxia (Licausi et al., 2010b). Support of a substantial role for HRE2 comes from the finding that ectopic overexpression of HRE2 with second residue mutated from Cys to Ala (C2A) increased seedling and plant tolerance to oxygen deprivation (Gibbs et al., 2011). Hence, the stress-

induced upregulation of HRE2 may have consequences that are distinct from RAP2.12. Thus, it is of interest to determine whether or not there is an overlap in the function of the N-end regulated proteins HRE2 and RAP2.12 in the context of the genes they regulate and the plant's acclimation to hypoxia.

Figure 1. Hypothesized model of the overlap in regulation by HRA1 and RAP2.12 under low oxygen stress based on current knowledge in *Arabidopsis*. Positive and negative regulations are depicted by “plus” and “minus” signs, respectively. Hypothesized regulations that will be tested or later proposed are indicated by question marks.



Submergence and anaerobic germination responses in rice

Group VII ERFs of rice are also relevant to survival of flooding in rice. Rice has a higher tolerance to flooding than other cereal species, but prolonged submergence has detrimental effects on the plant (Bailey-Serres et al., 2012). Both flooding and submergence are often accompanied by low oxygen stress. It has been shown that most rice cultivars die within one week of complete submergence due to carbohydrate starvation and an energy crisis (Fukao et al., 2006; Xu et al., 2006). Yet, there is genetic diversity in flooding tolerance in rice (Fukao et al., 2006; Xu et al., 2006; Bailey-Serres and Voesenek, 2008; Bailey-Serres et al., 2010a). Other plant species have higher susceptibility to flooding, but also have some natural genetic factors responsible for tolerance, such in *Arabidopsis* (Vashisht et al., 2011), *Glycine max* (soybean) (VanToai et al., 1994; Sayama et al., 2009), and *Rumex palustris* (marsh dock) (Vreeburg et al., 2005; Voesenek et al., 2006; Chen et al., 2011). Knowledge of the genetic variation for acclimation to flooding may aid the development of flooding tolerant plants.

Certain rice cultivars tolerate submergence through two strategies involving an ethylene-responsive pathway: by burning or economizing carbohydrate reserves. Rice seedlings of specific cultivars also display an anaerobic germination response by breaking down starch in a pathway involving *CALCINEURIN B-LIKE-INTERACTING PROTEIN KINASE15* (*CIPK15*, LOC_Os11g02240). *CIPK15* positively regulates a global energy and stress sensor, *SUC NONFERMENTING1 (SNF1)-RELATED PROTEIN KINASE1* (*SnRK1*, LOC_Os05g45420). By initiating the *SnRK1*-dependent sugar-sensing cascade to synthesize production of α -amylase and other hydrolases, and

catabolism of starch, seeds can germinate in the absence of oxygen under floodwater (Guglielminetti et al., 1995; Lee et al., 2009). In certain deepwater cultivars, rice in its vegetative stage of growth can undergo rapid elongation of the stem internodes until photosynthetic tissue is no longer under water. This “escape strategy” is determined by the *SNORKEL* (*SK*) locus, which encodes two ethylene-responsive group VII ERFs, *SK1* and *SK2* that triggers elongation of internodes, mediated by gibberellin (GA) (Hattori et al., 2009). The role of the *SKs* in gene regulation and GA biosynthesis or response awaits further elucidation.

On the other hand, rice can undergo submergence tolerance through a “quiescence strategy” in which energy is conserved until the floodwater recedes or reserves are exhausted (Fukao et al., 2006; Xu et al., 2006; Fukao and Bailey-Serres, 2008). It was discovered that a particularly submergence-tolerant rice landrace collected in eastern India, FR13A, was a potential genetic resource for breeding crops with increased submergence tolerance (Singh et al., 2001). The genetic basis of this tolerance was determined primarily by a quantitative trait locus (QTL) that mapped to the short arm of chromosome nine, and was named the *SUBMERGENCE 1* (*SUB1*) QTL (Xu and Mackill, 1996; Xu et al., 2000). Three group VII ERF transcription factors were found in the *SUB1* region of FR13A: *SUB1A* (DQ011598b), *SUB1B* (LOC_Os09g11480), and *SUB1C* (LOC_Os09g11460). These genes encode proteins that are 53 and 47% identical to SUB1A-1 at the amino acid level, respectively (Xu et al., 2006). This is primarily due to conservation of the ERF domain and N-terminal region, which is notably absent in SUB1C. The most extensive studies have focused on *SUB1A* since it was determined to

be necessary and sufficient for rendering a genotype lacking the gene submergence tolerance (Xu et al., 2006). The *SUB1B* and *SUB1C* genes are present in all *indica* and *japonica* varieties tested to date, but *SUB1A* is limited to a subset of *indica* varieties (Xu et al., 2006; Singh et al., 2009). Their function in the response to submergence is unknown, although Fukao et al. (2006) proposed that *SUB1A* acts upstream of *SUB1C*, a GA-responsive gene.

The molecular characterization of *SUB1* has enabled Asian rice cultivars with high grain yield and other desirable agronomic characteristics to be introgressed with the *SUB1* locus by marker-assisted backcrossing (Septiningsih et al., 2009). This “precision breeding” technique does not constitute the new “Sub1” varieties, such as Swarna-Sub1, as transgenic (e.g., genetically modified) rice. Sub1 rice has been rapidly adopted by farmers in regions with rice fields that are frequently inundated during monsoon rains. Rice genotypes bred to possess *SUB1A-1* show enhanced survival to the stress of submergence for 14 days or longer in greenhouse and field studies (Fukao et al., 2006; Septiningsih et al., 2009). The major function of *SUB1A* appears to be to restrict carbohydrate consumption, chlorophyll degradation, and elongation growth, resulting in avoidance of carbohydrate starvation during submergence. By economizing carbohydrate reserves under the stress, rice plants with *SUB1A* transcription factor can reinitiate formation of new leaves and recommence vegetative growth upon de-submergence (Fukao et al., 2006; Fukao and Bailey-Serres, 2008).

ADH and PDC are enzymes in the ethanol fermentation pathway that are activated during submergence, in which PDC breaks down pyruvate into acetaldehyde,

which is further catabolized to ethanol by ADH to recycle NAD^+ from NADH. NAD^+ is required for producing anaerobic energy through glycolysis, thus allowing the plant to utilize substrate level ATP production to avoid an energy crisis during submergence (Drew, 1997; Ismond et al., 2003; Fukao and Bailey-Serres, 2004; Bailey-Serres and Voesenek, 2008). *SUB1* lines display significantly higher upregulation of *ADH* and *PDC* mRNAs during submergence than near-isogenic lines, despite the limitation on carbohydrate consumption and ethanol production during the stress (Fukao et al., 2006). The overexpression of RAP2.12 in *Arabidopsis* was shown to induce expression of a promoter *ADHI*: luciferase reporter gene (Papdi et al., 2008). Thus, group VII ERFs may directly or indirectly regulate the conditional transcription of genes associated with anaerobic metabolism in both rice and *Arabidopsis*.

The *SUB1* locus of FR13A is polygenic, consisting of the three closely related ERF genes: *SUB1A-1*, *SUB1B-1*, and *SUB1C-1* (Xu et al., 2006). Allelic variation was recognized for each of these genes, with variation at *SUB1A* specific to phenotype. Under submerged conditions, the *SUB1A-1* allele showed much higher induction of transcript accumulation than the *SUB1A-2* allele (Xu et al., 2006; Septiningsih et al., 2008; Singh et al., 2009). The observation that the absence of *SUB1A-1* and low levels of *SUB1A-2* induction in submergence intolerant rice led to the testing of *SUB1A-1* as the submergence determinant in a transgenic plant (Xu et al., 2006). However, a subsequent survey of over 50 lines for *SUB1A* gene regulation and tolerance led to the finding that strong induction of *SUB1A-2* may also provide tolerance. Therefore, this study

considered the function of the *SUB1A-1* and *SUB1A-2* gene products as transcriptional activators.

Just as *HRA1* is proposed to be a negative regulator of transcription of *Arabidopsis* genes under hypoxia (Lee, 2012), phenotypic analysis and yeast-two-hybrid assays have similarly suggested that *SUB1A BINDING PROTEIN* (SAB18; LOC_Os11g06410) enhances submergence tolerance in rice (Seo et al., 2011). The trihelix DNA-binding protein SAB18 is a GT γ clade gene that is a rice relative of *HRA1* from *Arabidopsis*. Unlike *HRA1* of *Arabidopsis*, rice under submergence did not induce mRNA levels of *SAB18* (Jung et al., 2010; Mustroph et al., 2010). However, analysis of a transposon insertion allele of *SAB* suggested that this gene is important in regulation of submergence induced underwater elongation (Seo et al., 2011). It can be hypothesized that SAB18 of rice functions similarly to HRA1 in *Arabidopsis*.

Not all group VII ERFs may be substrates of the N-end rule pathway

Rice encodes an estimated 15 group VII ERFs (Sakuma et al., 2002; McGrath et al., 2005; Nakano et al., 2008). Based on sequence identity of the ERF and N-terminal domains, *SUB1A*, *SUB1B*, and *SK1* are group VII ERFs. Although *SUB1C* is most closely related to *SUB1A* and *SUB1B* (Fukao et al., 2009), it was not classified as a group VII ERF by Nakano et al. (2008) because of its lack of the conserved N-terminus. Interestingly, based on *in vitro* assay, the rice *SUB1* genes do not appear to be substrates of the N-end rule pathway mediated degradation (Gibbs et al., 2012). On the other hand, several other group VII ERFs were targets of the pathway *in vitro* (D. Gibbs, T. Fukao, J.

Bailey-Serres, and Michael Holdsworth, Nottingham University and UC Riverside, unpublished data).

Aims of this study

The purpose of this thesis research project was to examine the functional roles of transcription factors of the ethylene response factor (ERF) group VII in rice and *Arabidopsis*. As described earlier, the group VII ERFs play significant roles in the submergence tolerance of rice (Fukao et al., 2006; Xu et al., 2006), and in survival of low oxygen conditions in *Arabidopsis*. There is evidence that group VII ERFs directly or indirectly regulate genes encoding enzymes important in anaerobic metabolism in both rice and *Arabidopsis*. The primary goal was to examine whether group VII ERFs positively regulate core hypoxia responsive genes in both species. This provides the first step in determining whether the same direct gene targets or *cis*-element(s) are controlled by the group VII ERFs *SUB1A-1*, *SUB1A-2*, *SUB1B-1*, *SUB1C-1*, *HRE2*, and *RAP2.12*. The secondary goal was to examine the relationship of the group VII ERFs with HRA1, a trihelix protein that negatively regulates many of the core hypoxia responsive genes of *Arabidopsis*.

Experimental approach to examine direct targets of group VII ERFs

DNA microarray or mRNA-seq analysis can identify differentially regulated genes by comparison of samples (i.e., condition, developmental stage, genotype) and expose at the genome-wide level the genes that are upregulated or downregulated. For example, Lee et

al. (2010) performed DNA microarray hybridizations to compare changes in transcript abundance in root and shoot tissue in response to submergence, complementing earlier evaluation of seedling responses to oxygen deprivation (Mustroph et al., 2010 and references therein). These studies identified an overlap of 34 genes under submergence and low oxygen conditions (Lee et al., 2011). Of these, 10 were shown to be differentially regulated by RAP2.12 (Lee et al., 2011; Licausi et al., 2011). Based on these data, *ADH1*, *PDC1*, *HRE1*, *HRE2*, and *HRA1* are hypothesized to be in a regulatory network with RAP2.12 shown in Figure 1. These findings raise the question whether group VII ERFs directly or indirectly regulate the condition-specific increases in these genes. Since transcript accumulation studies, rendered by DNA microarray, mRNA-seq, or quantitative real time polymerase chain reaction (qRT-PCR) does not reveal direct transcriptional activation, additional approaches *in vitro* or *in vivo* must be made to test the relationship between these transcription factors and genes.

There are several methods to examine the targets of group VII ERFs, but all have their advantages and drawbacks. Chromatin immunoprecipitation assays combined with DNA tiling arrays (ChIP-chip) or high-throughput sequencing (ChIP-seq) could be performed to investigate the direct binding of *cis*-acting targets by specific transcription factors. ChIP is an ideal initial process for distinguishing gene targets of a transcription factor, but may require subsequent demonstration of direct versus indirect DNA interaction. This *in vivo* assay involves cross-linking DNA to binding proteins, including transcription factors and histones. The protein of interest is then immunoprecipitated from the sheared chromatin preparation using an antibody that is specific to the DNA

binding protein of interest (Collas, 2010). Although this approach can identify which genes are regulated by a transcription factor of interest, the specificity is limited to the amount of protein crosslinking to the protein-DNA complex and cannot be used to presume actual transcriptional activity (Park, 2009). Preliminary ChIP-seq data identified a small number of potential *HRA1* targets during hypoxia in *Arabidopsis* seedlings (Lee, 2012) but ChIP-seq identification of HRE2 targets was less clear (Lee, S.C., and Bailey-Serres, J., unpublished). ChIP only reveals interacting protein and DNA, but gives no information on whether the protein association with a chromatin region corresponds to activated, repressed, or silenced genes.

ChIP-chip and ChIP-seq identifies transcription factor binding sites at the genome wide level, but yeast-one-hybrid assay (Y1H) can similarly identify direct binding of proteins to DNA elements of interest with more focus on a particular set genes or their regulatory regions (i.e., 5' flanking regions). Y1H involves cloning of a hybrid protein that fuses the “bait” DNA sequence of interest upstream of a reporter gene with a promoter. The library of proteins to be tested is cloned into a vector containing a *trans*-activation domain, such as the yeast GAL4 transcription factor. Transcription of the reporter gene is activated when the protein interacts with the sequence of interest at the target site, either through direct binding to the DNA or indirect binding by interactions with a DNA-binding protein (Alexander et al., 2001; Hens et al., 2011; Ouwerkerk and Meijer, 2011). This method is useful when specific antibodies for particular binding sites are not available for ChIP assays. However, there may be possible false positives due to the use of an artificial system in yeast. When a protein is artificially placed into the yeast

nucleus, highly charged regions of the protein can cause precocious transcription of the reporter gene. There are also false negatives due to steric hindrance from fused yeast reporter proteins that impede interaction when expression should have occurred. Transcription factors encoded by long open reading frames or expressed at extremely low levels may not be represented in cDNA libraries (Alexander et al., 2001).

The gel electrophoresis mobility shift assay (EMSA) is also used to study the interaction of transcription factors to specific DNA sequence *in vivo*. This technique is based on the fact that the electrophoretic mobility of a protein-nucleic acid complex is typically less than that of the free nucleic acid (Hellman and Fried, 2007). A short region of DNA hypothesized to contain a transcription factor binding site is radiolabeled, incubated with the protein of interest, and the protein-DNA mixture is fractionated in a polyacrylamide or agarose gel. If protein(s) is bound to the DNA fragment, it will be less mobile and thus separated from the fragment without the protein(s). DNA-protein interactions need to be validated by competition with non-labeled oligonucleotides, often containing a putative *cis*-acting element. EMSA only requires a small amount of protein and nucleic acid, and is a relatively straightforward method used to indicate if one or more protein is involved in a binding complex. Typically, the method uses protein produced in a heterologous system, such as in *E. coli*. Because of this, a downside of the method is that the protein may not have post-translational modifications that are present or regulated in the plant cell. EMSA could confirm the mode of interaction of RAP2.12 and HRA1 on the *PDC1* and *HRA1* anaerobic promoters using defined probes for regions of the *PDC1* and *HRA1* promoters. Yet, EMSA is prone to false results due to *in vitro*

optimization and requires high resolution, so this method should accompany other experimental approaches.

Transient protoplast transfection is another approach to examine direct regulation of the group VII ERF transcription factors. Protoplasts are plant cells that have their cell wall entirely removed by the polysaccharide-degrading enzymes cellulase and macerozyme. By using the protoplast transfection system, analysis of genes can be completed relatively rapidly and efficiently as compared to other methods (Yoo et al., 2007; Zhang et al., 2011). Plasmid DNA is delivered into the protoplasts by PEG–calcium-mediated transfection, and a 16–20 h incubation primes the protoplasts for detection of gene expression. Measure of luciferase activity is typically used to determine whether a transcription factors drives activation or repression of a specific promoter linked to a luciferase coding sequence. Using protoplasts derived from plant material provides more functional and physiological relevance as compared to using other systems such as yeast in Y1H assays. The protoplast system can detect both direct and indirect gene expression regulation since the cells contain all other essential genes that are required for expression of a reporter gene. However, this system cannot detect direct interactions between cells since protoplasts lack a cell wall. The plasmodesmata and other intracellular connections are severed, thus abrogating the cell-to-cell signaling. Studies of the functional regulations of group VII ERFs will be performed through protoplast transient transfections in this thesis.

Table 1. Comparison of methods for examining direct regulation by a transcription factor.

Method	Summary of Method	Level of Detecting Interaction	Advantages	Disadvantages
Chromatin Immunoprecipitation (ChIP)	DNA-binding proteins crosslinked to the DNA binding site are immunoprecipitated with antibodies specific for the proteins	Directly identifies the cistrome of DNA-associated proteins or indirectly identifies TFs associating with DNA through interaction with other proteins	Provides <i>in vivo</i> targets for further analysis; ChIP-seq generates large quantities of data	Can have low sensitivity or specificity; need specific antibodies; high cost for quantitative PCR or sequencing
Yeast-One-Hybrid (Y1H)	DNA sequence of interest is fused to a reporter gene and integrated into a yeast genome; cell-cell mating occurs between the reporter yeast strain and a strain with a protein library containing a GAL4 transcription activation domain	Allows for screening of novel protein partners with a DNA bait and identifies direct binding of proteins to <i>cis</i> -acting DNA elements of interest	Low cost; large-scale output; no need for optimization of <i>in vitro</i> conditions	Instances of false positives or negatives; large scale yeast transformation can be laborious
<i>In vitro</i> Electrophoretic Mobility Shift Assay (EMSA)	Labeled DNA- protein complexes formed <i>in vitro</i> and are electrophoretically separated on a gel from free nucleic acid; often utilizes <i>E. coli</i> -produced proteins or nuclear extracts	DNA oligonucleotide probes assess the degree of affinity or specificity of the transcription factor interactions to specific DNA sequence, but does not detect direct interactions	Easy to perform; sensitive enough to detect low abundance DNA binding proteins from lysates	Difficult to quantitate; dissociation of protein complexes can occur during electrophoresis; does not account for <i>in vivo</i> protein modifications
Protoplast Transient Transfection	Protoplasts are isolated from plant tissue and subjected to PEG–calcium-mediated transfection with reporter genes	Luciferase reporter assays reveal direct or indirect transcription factor–promoter interactions; cannot detect direct interactions between cells	Cheaper and more rapid biological system for gene expression analysis	Protoplast yield and high transfection efficiency is dependent upon specific growth conditions

Abbreviations: PCR, polymerase chain reaction; PEG, polyethylene glycol; TF, transcription factor.

Other methods to study interaction between two transcription factors

Interactions between two transcription factors can be further examined with other experimental approaches. Co-immunoprecipitation (Co-IP) assays can identify direct interacting partners that interact with a specific group VII ERF gene, which can give further insight into the functional and cellular activities of the gene. An antibody against a specific target protein forms a complex with that target in a cell lysate, which is precipitated on a bead support. After elution of the affinity purified protein complex, analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot detection serve to verify the antigen identity (Phizicky and Fields, 1995). Alternatively, the immunopurified proteins may be determined by mass spectrometry. Others are involved in probing for products interacting with the ERFs under hypoxia stress using α -FLAG or α -HA epitope-tagged versions of the group VII ERF genes (Travis Lee, University of California, Riverside, USA, personal communication).

A yeast-two-hybrid (Y2H) assay is similar to the Y1H system but identifies protein-protein interactions instead of protein-DNA interactions. A downstream reporter gene is activated by a transcription factor binding to an upstream activating sequence (UAS) (Joung et al., 2000). The transcription factor is split into a binding domain for UAS-binding and an activating domain for activating transcription. Although this method allows for initial identification of interacting partners, Y2H is subjected to false positives and negatives.

Co-transient transformation can also reveal interactions between two transcription factors. The CaMV 35S promoter-driven expression vector is co-transformed with two transcription factors into protoplasts. Upon identification of the direct targets of the transcription factors and their interactions with one another, confirmations by *in planta* studies should be performed. One approach is to breed transgenic *Arabidopsis* and rice plants with mutated *cis* elements so the functional loss of the elements can be studied. For example, the anaerobic response elements (ARE) in transgenic plants were studied using protoplasts of maize (Olive et al., 1990). Another tactic is to test a species' ERFs involved in low oxygen responses in the same species and in other organisms. For example, a rice ERF might be tested in both rice and *Arabidopsis* protoplasts.

Once a transcription factor and its target sequence is identified using protoplast transfection, EMSA, or Y1H, and initial validation of a binding sequence is confirmed (i.e., by mutation of the putative *cis*-element and analysis these systems), *in planta* confirmation can be obtained using stably transformed plants (Bent, 2000; Fauser, 2012). Genotyping of plant inbred lines and other molecular analyses can give more insight into the functional characteristics of the ERFs.

It is anticipated that this study of the conserved group VII ERF transcription factors will provide more insight into the molecular mechanisms involved in energy management underlying abiotic stress tolerance in *Arabidopsis* and rice. Experiments performed in this study confirmed targets of RAP2.12 and HRE2 in *Arabidopsis*, and identified *SUB1A-1*, *SUB1A-2*, *SUB1B-1*, and *SUB1C-1* targets in rice. The group VII ERFs of both species showed some conservation of gene regulation in the core response

to low oxygen conditions. This further elucidation of the group VII ERF gene networks along with the regulation of their proteolytic turnover, will facilitate development of new crop species with robust stress tolerance.

Chapter 2

MATERIALS AND METHODS

2.1 Plant materials and growth conditions

Arabidopsis thaliana (Columbia (Col-0); *Arabidopsis* Biological Resource Center) and *Nicotiana benthamiana* were used. Seeds were surfaced-sterilized, imbibed in the dark for 3 d at 4°C, and germinated on agar media plates. Plants were transferred to soil and grown in an environment-controlled chamber with a short photoperiod (10–13 h light at 23°C/11–14 h dark at 20°C) under low light ($50\text{--}75\text{ mE m}^{-2}\text{ s}^{-1}$) and 65% relative humidity. Three to four weeks old plants were used for protoplast isolation.

Oryza sativa japonica cv. M202 was used for isolation of rice protoplasts. M202 lacks *SUB1A*. Sterilized seeds were germinated on wet paper towels for 6 d at 25°C under fluorescent light. The germinated seedlings were transplanted to soil in pots (J.M. McConkey & Co, Inc, Sumner, WA, USA; $4 \times 4 \times 3.5$ in) and grown for 10 d in a greenhouse under natural light as described previously (Fukao et al., 2006).

2.2 Construction of plasmids

Plasmids were constructed using PCR-based cloning. Rice genes were amplified from M202 genomic DNA using specific forward and reverse primers (Table 1) in a 50 μL PCR reaction mixture [10 μL of 5 $\times$ HotStar HiFidelity PCR Buffer PCR (1 $\times$ final concentration), 10 μL of 5 $\times$ Q-Solution (1 $\times$ final concentration), 5 μL each of a 10 μM forward and reverse primer (1 μM final concentration), 1 μL of HotStar HiFidelity DNA

Polymerase (2.5 units final concentration), 10 ng of template DNA, and RNase-free Water up to a total of 50 μ L volume]. The PCR thermal cycler conditions for amplifying the DNA are as follows: An initial 5 min denaturation step at 95°C followed by ~30 thermal cycles each consisting of denaturation at 94°C for 5 min, annealing at 5°C below T_m of the primers for 1 min, and extension at 72°C for 1 min. The reaction ended with a final extension of 10 min at 72°C after the last cycle. At the end of cycling, the reaction was incubated at 4°C infinitely until further use. The amplified PCR product was isolated on a 1.5% agarose gel and purified using a MinElute Gel Extraction Kit (Qiagen, Hilden, Germany). The DNA insert was then ligated into a pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA, USA). Using the Gateway LR Clonase II enzyme mixture (Invitrogen), the entry vector containing the gene of interest flanked by two *attL* sites was recombined into binary Gateway destination vectors carrying two *attR* sites in a standard Gateway cassette (*attR1-ccdB-attR2*) (Karimi et al., 2007). The Gateway destination vectors used were: p2GW7 [a plasmid in a pPZP200 vector backbone with an ampicillin selectable marker for overexpression of a DNA sequence; the Gateway cassette site was placed between the promoter and the terminator of the cauliflower mosaic virus (CaMV) 35S transcript]; p2FGW7 [a plasmid with ampicillin selection useful for examining localization of particular protein under control of the CaMV 35S promoter placed upstream of the 5' untranslated region of the gene of interest; downstream of the CaMV 35S promoter is the Gateway cassette site which enabled translational fusion of a coding sequence with GFP at the N-terminus, followed by the 35S 3' terminator sequence]; p2GWF7 (a plasmid with ampicillin selection also used for examining protein localization under the

heterologously strong CaMV 35S promoter placed directly upstream of a coding sequence with GFP at the C-terminus; downstream of this GFP protein is a Gateway cassette site for translational fusion of the 5' untranslated region of the gene of interest, followed by the 35S 3' terminator sequence]; and LUC SMALL [a firefly luciferase reporter vector in the p2GW7 overexpressor backbone with a Gateway entry cassette for fusion to the promoter of a gene of interest] LUC. (Karimi et al., 2002, 2007). These vectors were transformed into OneShot Top 10 competent cells (Invitrogen) or DB3.1 competent cells and purified using a QIAprep Spin Miniprep Kit (Qiagen). The HiSpeed Plasmid Maxi Kit (Qiagen) was used to obtain sufficient quantities of purified plasmid DNA (650–1100 µg/ul) for protoplast transformation. All plasmids were solubilized in water and stored at -20°C. Plasmid constructs were confirmed by Sanger DNA sequencing using primers listed in Table 2. All plasmids used in this study are listed in Table 3.

Table 2. Sequences of forward (F) and reverse (R) primers used in this work for constructing and sequencing plasmids.

Primer Name	Sequence (5'→ 3')	T _m (Melting Temp)	Comments
35S Terminator-R	R: GAG AGA CTG GTG ATT TTT GC	—	primer to sequence insertions from the terminal end of the CaMV 35S promoter
35S Promoter-F	F: CTA TCC TTC GCA AGA CCC TTC	—	primer to sequence insertions upstream of the CaMV 35S promoter
α Amy3D promoter	F: CAC CCT CCA CTG CTC CAT AG R: CGT CCA GGT CGT AGA GAC	54°C 54°C	primers for amplifying the α Amy3D promoter region in rice
ADH1 promoter	F: CAC CAG CGA ACG TGA TGC CTC CTT TC R: TAC TCT CCT CCC CAC AAA ATC CCG CAC	64°C 64°C	primers for amplifying the ADH1 promoter region in rice
CIPK15 promoter	F: CAC CAT GCA GCT TAA CTC C R: CAG CAC TAT GTT TTG ACC AC	54°C 52°C	primers for amplifying the CIPK15 promoter region in rice
LUC-R	R: CTC TAG AGG ATA GAA TGG CGC	—	primer to sequence insertions upstream of the luciferase gene
M13-F (-20)	F: TGT AAA ACG ACG GCC AGT	—	primer to sequence gene insertions in the pENTR vector
M13-R	R: GAA ACA GCT ATG ACC ATG AT	—	primer to sequence gene insertions in the pENTR vector
SAB18	F: CAC CAT GTC TAT CCT CCT TTG R: CAT GGA TGA TTA GCA TTG CCC	53°C 54°C	primers for amplifying the SAB18 gene in rice
SUB1B-1	F: ATG TGT GGA GCA CTG ATC R: CTA ATT AAC CAT TCC CAA AAT A	59°C 56°C	primers for amplifying the SUB1B-1 gene in rice
Ubi-F	F: TGA TAT ACT TCG ATG ATG GCA	—	primer to sequence insertions downstream of the maize Ubiquitin1 promoter

Table 3. List of all plasmids used in this study. The locus identifier for *Oryza sativa* genes were identified by the Michigan State University Rice Genome Annotation Project (MSU; <http://rice.plantbiology.msu.edu>). If the gene is absent from the Nipponbare subspecies of rice and thus has no LOC number, it is identified by the GenBank accession number from the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov/genbank). *Arabidopsis thaliana* genes are identified based on their *Arabidopsis* Genome Initiative (AGI) accession number from The Arabidopsis Information Resource database (TAIR; www.arabidopsis.org).

Plasmid	Os Locus/GenBank/ ATG number	Purpose	Description/Comments	Bacterial Resistance	Source	References
<i>promOsADH1:PpLUC</i>	LOC_Os11g10480	reporter	promoter region of <i>ADH1</i> fused to luciferase for luciferase activity detection	amp ^R	This study	Fukao et al., 2006, 2011; Jung et al., 2010
<i>promOsCIPK15:PpLUC</i>	LOC_Os11g02240	reporter	promoter region of <i>CIPK15</i> fused to luciferase for luciferase activity detection	amp ^R		Bailey-Serres and Voesenek, 2008; Lee et al., 2009
<i>promOsPDC1:PpLUC</i>	LOC_Os01g06660	reporter	promoter region of <i>PDC1</i> fused to luciferase for luciferase activity detection	amp ^R		Fukao et al., 2006
<i>35S:SAB18</i>	LOC_Os11g06410	effector	overexpressed <i>SAB18</i> under control of the 35S CaMV promoter	amp ^R		Jung et al., 2010; Mustroph et al., 2010; Seo et al., 2011
<i>35S:SUB1B-1</i>	LOC_Os09g11480	effector	overexpressed <i>SUB1B-1</i> under control of the 35S CaMV promoter	amp ^R		Fukao et al., 2006, 2009, 2011, 2012; Bailey-Serres et al., 2010
<i>p2FGW7:SUB1A-1</i>	DQ011598b	subcellular localization vector	overexpressed <i>SUB1A-1</i> with N-terminally tagged GFP under control of the 35S CaMV promoter	amp ^R		
<i>p2GWF7:SUB1A-1</i>	DQ011598b	subcellular localization vector	overexpressed <i>SUB1A-1</i> with C-terminally tagged GFP under control of the 35S CaMV promoter	amp ^R		
<i>p2FGW7:SUB1C-1</i>	LOC_Os09g11460	subcellular localization vector	overexpressed <i>SUB1C-1</i> with N-terminally tagged GFP under 35S CaMV promoter control	amp ^R		

Plasmid	Os Locus/GenBank/ ATG number	Purpose	Description/Comments	Bacterial Resistance	Source	Reference
<i>p2GWF7:SUB1C-1</i>	LOC_Os09g11460	subcellular localization vector	overexpressed <i>SUB1C-1</i> with C-terminally tagged GFP under control of the 35S CaMV promoter	amp ^R	This study	Fukao et al., 2006, 2009, 2011, 2012; Bailey-Serres et al., 2010
<i>35S:SUB1A-1</i>	DQ011598	effector	overexpressed <i>SUB1A-1</i> under control of the 35S CaMV promoter	amp ^R	Takeshi Fukao and Julia Bailey- Serres , UC Riverside, unpublished	
<i>35S:SUB1A-2</i>	DQ011598	effector	overexpressed <i>SUB1A-2</i> under control of the 35S CaMV promoter	amp ^R		
<i>35S:SUB1C-1</i>	LOC_Os09g11460	effector	overexpressed <i>SUB1C-1</i> under control of the 35S CaMV promoter	amp ^R		
<i>promAthHRA1:PpLUC</i>	At3g10040	reporter	promoter region of <i>HRA1</i> fused to luciferase for luciferase activity detection	amp ^R	Beatrice Giuntoli and Pierdomenico Perata , Scuola Superiore Sant'Anna, Italy, unpublished	Lee, 2012
<i>35S:HRA1</i>	At3g10040	effector	overexpressor of <i>HRA1</i>	amp ^R		
<i>35S:HRA1Δ322</i>	At3g10040	effector	overexpressor of truncated <i>HRA1</i> with a C-terminal deletion (residues proceeding the 322nd are truncated)	amp ^R		
<i>35S:Δ193HRA1</i>	At3g10040	effector	overexpressor of <i>HRA1</i> with a N-terminal deletion (first 193 residues are truncated)	amp ^R		
<i>promAthPDC1:PpLUC</i>	At4g33070	reporter	promoter region of <i>PDC1</i> fused to luciferase for luciferase activity detection	amp ^R		Lee et al., 2011
<i>35S:Δ13RAP2.12</i>	At1g53910	effector	overexpressed <i>RAP2.12</i> with first 13 N-terminal residue deletions under 35S CaMV promoter control	amp ^R		Gibbs et al., 2011; Licausi et al., 2011; Lee, 2012

Plasmid	Os Locus/GenBank/ ATG number	Purpose	Description/Comments	Bacterial Resistance	Source	Reference
<i>35S::C2A-HRE2</i>	At2g47520	effector	overexpression of the mutated (C2A) form of <i>HRE2</i>	amp ^R	Michael Holdsworth and Daniel Gibbs , University of Nottingham, England	Gibbs et al., 2011; Lee, 2012
p2GW7	—	entry vector	35S::gateway cassette in a small backbone vector	kan ^R	VIB Dept of Plant Systems Biology Dept , Ghent University, Belgium	Karimi et al., 2002, 2003, 2007
p2FGW7	—	entry vector	35S::GFP::gateway cassette in a small backbone vector	kan ^R		
p2GWF7	—	entry vector	35S::gateway cassette::GFP in a small backbone vector	kan ^R		
LUC SMALL	—	entry vector	gateway cassette::LUC in a small backbone vector; constructed by recombination of cassette:LUC::t35S fragment from pBGWL7 into the small backbone of p2GW7	kan ^R	Francesco Licausi , Scuola Superiore Sant'Anna, Italy	van Leeuwen et al., 2000
<i>pENTR</i> D-TOPO	—	entry vector	entry vectors used for ligation reaction cloning to transfer the insert from entry clones to its destination vectors	kan ^R	Invitrogen , Darmstadt, Germany	<i>pENTR</i> Directional TOPO Cloning Manual, Invitrogen

2.3 Protoplast isolation

Arabidopsis protoplast isolation was performed according to the protocol from Yoo et al. (2007). For isolation of *Arabidopsis* protoplasts, the fifth to seventh leaves from 3–4-week-old plants were cut with a sharp razor blade into 0.5–1 mm strips. The strips were immediately submerged into an enzyme solution [20 mM 4-morpholineethanesulfonic acid (MES; pH 5.7), 1.5% (w/v) cellulase R10 (Yakult Pharmaceutical Ind. Co., Ltd., Japan), 0.4% (w/v) macerozyme R10 (Yakult Pharmaceutical Ind. Co., Ltd., Japan), 0.4 M mannitol, 20 mM KCl, 10 mM CaCl₂, and 0.1% (w/v) Bovine Serum Albumin] in a 50 mL Falcon tube. The leaf strips were vacuum infiltrated in the dark for 30 min using a PVC vacuum tube connected to a rubber stopper, and the digestion was continued in the dark for at least 3 h at 23–25°C. An equal volume of W5 solution [2 mM MES (pH 5.7), 154 mM NaCl, 125 mM CaCl₂, and 5 mM KCl] was added. Protoplasts were filtered through a 40 µm nylon mesh into 30 mL round-bottomed tubes. A protoplast pellet was collected by centrifugation at $100 \times g$ for 2 min. After counting cells using a hemacytometer on an inverted microscope, the pellet was resuspended at 2×10^5 cells mL⁻¹ in W5 solution. The protoplasts were set on ice for 30 min or until they settled to the bottom of the tube. The W5 solution was then replaced with MMG solution [4 mM MES (pH 5.7), 0.4 M mannitol, and 15 mM MgCl₂] at 23–25°C.

The same protocol was used for rice protoplast isolation, with modifications adapted from Bart et al. (2006), Chen et al. (2006), and Zhang et al. (2007). Green tissues from the stem and sheath of 100 10-d-old rice seedlings were used. Enzymatic digestion was carried out for 4 to 5 h in the dark with gentle shaking (60–80 rpm) without vacuum

infiltration. The mannitol concentration of the W5 was increased to 0.6 M. The protoplasts were resuspended in W5 solution at $5\text{--}8 \times 10^5$ cells ml^{-1} .

2.4 Protoplast transfection

Three plasmids expressing a regulatory effector (transcription factor), a specific reporter (promoter:reporter) and a transfection control reporter (*35S:Renilla LUC*) were mixed in a 2-ml microfuge tube in ratio of 5:4:1 (Yoo et al., 2007). One hundred microliter (100 μl) of protoplasts (2×10^4 *Arabidopsis* protoplasts and 8×10^4 rice protoplasts) were added and gently mixed with 30% polyethylene glycol (PEG) [0.2 M mannitol, 100 mM CaCl_2 , 30% (w/v) PEG-4000] and incubated at 23–25°C for 8 min. The transfection process was stopped by addition of 400 μl of W5, and centrifuged for 2 min at $100 \times g$. The pellet was resuspended gently in 0.25 mL WI solution [0.5 M 4 mM MES (pH 5.7), 0.5 M mannitol, and 20 mM KCl] and the protoplasts were transferred into 24-well plates coated with 5% calf serum and then incubated under continuous light at room temperature for 16–20 h. Protoplast harvest was conducted by collection into 2 mL centrifuge tubes with a wide bore pipette and centrifugation at $100 \times g$ for 2 min. The supernatant was removed and the samples were frozen in liquid nitrogen and stored at -80°C until further use.

2.5 Dual-luciferase assay

Luciferase activity was quantified using the Dual-Luciferase Assay System (Promega, Madison, WI, USA) and a TD-20/20 luminometer (Turner Designs, Inc., Sunnyvale, CA,

USA). Freshly prepared cold lysis buffer [2.5 mM Tris-phosphate (pH 7.8) containing 1 mM dithiothreitol (DTT), 2 mM trans-1,2-diaminocyclo-hexane-N,N,N',N'-tetraacetic acid (DACTAA), 10% (v/v) glycerol and 1% (v/v) Triton X-100] was added to the protoplasts. The cells were lysed by vortexing. Samples were centrifuged and 20 µl of the supernatant was mixed with 100 µl of Luciferase Assay Reagent II (firefly luciferase; Promega), and luminescence was read for 10 sec with a 2 sec delay. To monitor *Renilla reniformis* (Renilla) luciferase, 100 µl of Stop and Glo buffer (Promega) was immediately injected and luminescence was read for another 10 sec with a 2 sec delay.

2.6 Fluorescence imaging of GFP and nuclear DNA

Protoplasts were transfected with N- or C-terminally tagged GFP fusion constructs. Fluorescence of live cells was viewed by microscopy (Leica DM LB2; Spot Advanced software) using the appropriate filter with a UV excitation range for DAPI fluorophore (BP 340-380 nm excitation filter and LP 425 emission filter) or with a blue excitation range for GFP (BP 450-490 nm excitation filter and LP 515 emission filter). Fluorescence images were captured at 40× or 100× magnification. Nuclei were stained using 4', 6-diamidino-2-phenylindole (DAPI) diluted to 300 nM in W5 solution. To a 100 µl suspension of 2×10^5 *Arabidopsis* protoplasts, 200 µL of the DAPI staining solution was added, ensuring complete coverage, and incubated at 23–25°C for 5 min. After three washes with W5 solution, protoplasts were viewed using a fluorescence microscope and photographed.

Chapter 3

RESULTS

Strict regulation of transcription is dependent upon the interaction of *cis*-acting DNA regulatory elements with *trans*-acting transcription factors. DNA microarray and other gene expression analyses using genetic combinations with altered levels of group VII ERFs indicates that these putative transcription factors drive the expression of core hypoxia responsive genes (Fukao et al., 2006; Xu et al., 2006; van Dongen et al., 2009; Jung et al., 2010; Mustroph et al., 2010; Lee et al., 2011; Licausi et al., 2011; Gibbs et al., 2011; Peña-Castro et al., 2011; Yang et al., 2011). The hypothesis addressed here is that group VII ERFs regulates gene regulatory networks associated with anaerobic metabolism in both *Arabidopsis* and rice. This was addressed using transient transformation of *Arabidopsis* and rice protoplasts.

3.1 Dual luciferase system as a method of detection

Transient gene expression data provided insight into promoter activities and subcellular localization. Essentially, the protoplast transfection system measures the ability of an effector plasmid expressing a transcription factor to activate a given promoter (Figure 2). Here, protoplasts were transformed with varying concentrations of a plasmid to evaluate dose-dependent regulation of the reporter construct. To avoid confusion between genes of *Arabidopsis* and rice that were used as the reporters of gene expression, the promoter constructs indicate the plant of origin (i.e.,

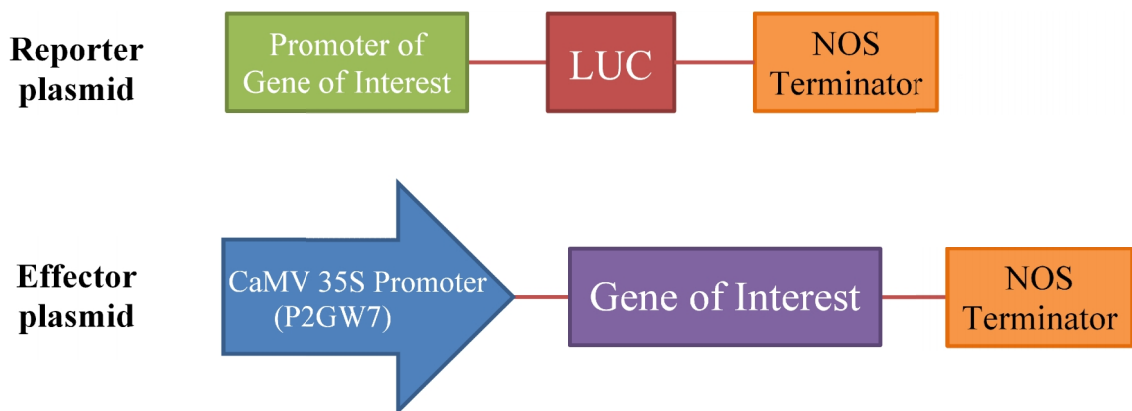
promAthPDC1:PpLUC and *promOsPDC1:PpLUC* correspond to the promoters of the *PDC1* gene of *Arabidopsis* and rice, respectively.

The transient gene expression system used in this study consists of three parts: the host vectors, the gene delivery vectors, and a luciferase detection method. Luciferase activity is a common method of detection for living protoplasts in the transient expression system. *35S:Renilla LUC* (*35S:RrLUC*) serves as an internal control for normalizing the transformation efficiency. In the study, the gene transfer in the effector plasmids was monitored using a dual-luciferase system. A luminometer detected the flash of light from the protoplast extract that express a *promoter:LUC* reporter gene in the presence of a firefly luciferase enzyme that oxidizes beetle luciferin to oxyluciferin in the presence of ATP, oxygen, coenzyme A and Mg^{2+} . After a 2 second delay and a 10 second reading measurement, the reaction was quenched, and a reagent was added to catalyze a luminescent reaction involving the conversion of a coelenterazine substrate to coelenteramide by Renilla luciferase. The luminometer measured the relative light units (RLU), which is proportional to the amount of luciferase activity. The data analyzed was based on comparison of the firefly RLU / Renilla RLU signal ratio.

For some experiments, there were varying values in Renilla RLU, as much as a 100-fold difference. This variability was the main reason for the high standard deviations in the pool of three technical replicates. The cause of the variability may be due to the different level of stress in the protoplasts during each technical replicate of the protoplast transfection. Hence, the amount of dead cells was monitored in each experiment using the vital stain Evan's blue, before and after the overnight incubation of the protoplasts. In the

reported experiments, the number of dead protoplasts between technical replicates was not significantly different. Another possible reason for the disparity between biological replicates was that a variable number of protoplasts may have been used between transfections since the protoplasts were freely suspended in a solution. Because of this challenge, in a few cases the data are representative rather than means of pooled biological replicates. In these cases, further confirmation of the results would be warranted.

Figure 2. Schematic illustration of the effector and reporter constructs used in the protoplast transient expression studies. Luciferase expression vectors are expressed by use of the quasi-constitutive CaMV 35S promoter, and luciferase (*LUC*) is driven by the promoter of the gene of interest. The effector construct is overexpressed under control of the CaMV 35S promoter driving the gene of interest.



3.2 Functional study of 35S:C2A-HRE2 in *Arabidopsis* using *HRA1* and *PDC1* promoters

HRE2 is proposed to target expression of core hypoxia genes. Mutation of the second Cys residue of *HRE2* to Ala stabilizes the HRE2 protein under normoxic conditions, allowing it to escape N-end rule degradation. To test the activity of the stabilized HRE2, it was co-transfected into protoplasts with LUC fusion constructs for two anaerobic response genes: *HYPOXIA RESPONSE ATTENUATOR 1* (*promAthHRA1:PpLUC*) and *PYRUVATE DECARBOXYLASE 1* (*promAthPDC1:PpLUC*). The effect of 35S:C2A-HRE2 on transactivation of the *HRA1* and *PDC1* promoters was examined using different quantities of the effector plasmid DNA.

The data revealed that 35S:C2A-HRE2 increases expression of *promAthHRA1:PpLUC* and *promAthPDC1:PpLUC* in a dose-dependent manner. There was low transactivation of luciferase in the non-transfected control, with increasing relative luciferase activity from 0.5 to 6 µg of the 35S:C2A-HRE2 (Figures 3 and 4). These dose-dependent responses are consistent with the transactivation of these promoters observed with RAP2.12 (B. Guintoli, personal communication of unpublished data). To address whether there might be an interaction between the two transcription factors HRE2 and HRA1, the 35S:C2A-HRE2 effector was transfected in conjunction with the 35S:HRA1 construct (Figure 5). It was observed that 35S:HRA1 did not transactivate its own promoter, whereas 35S:C2A-HRE2 activated *promAthHRA1:PpLUC*. Interestingly, 35S:HRA1 inhibited 35S:C2A-HRE2 promotion of the *HRA1* promoter, with the highest reduction in transactivation at 6 µg 35S:HRA1.

However, this inhibition by *35S:HRA1* was not detected in the *35S:C2A-HRE2* activation of the *PDC1* promoter. *35S:HRA1* instead elevated *35S:C2A-HRE2* promotion, which in turn activated the *PDC1* promoter (Figure 6). *35S:HRA1* alone also increased *promAthPDC1:PpLUC* expression, a trend that was not observed with *promAthHRA1:PpLUC*. This result is opposite from that observed with RAP2.12, in which co-transfection of *35S:HRA1* reduced transactivation (B. Guintoli, unpublished data). This induction was not expected, so there may be an alternative regulatory mechanism for HRE2 and HRA1.

Figure 3. HRE2 transactivates the *HRA1* promoter. Transient transfection of varying amounts of *35S:C2A-HRE2* demonstrates activation of *promAthHRA1:PpLUC* in *Arabidopsis* protoplasts. Twenty-thousand protoplasts were transfected with 4.8 μ g *promAthHRA1:PpLUC* and 1.2 μ g *35S:Renilla LUC* plasmid DNA as well as the amount specified of *35S:C2A-HRE2* plasmid. LUC activities were determined after 16 h of culture. Values are plotted for the mean of three independent biological replicates, each with three technical replicates. Error bars represent $\pm$ SD.

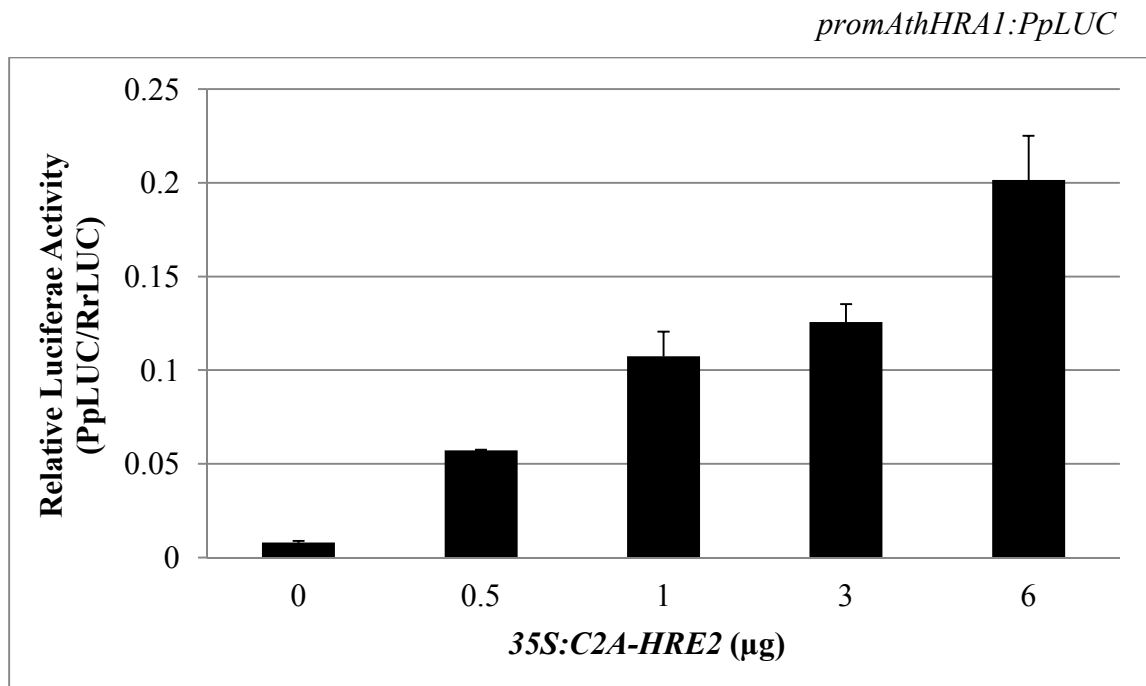


Figure 4. HRE2 activates the *Arabidopsis PDC1* promoter in *Arabidopsis* protoplasts.

Twenty-thousand protoplasts were transfected with 4.8 μg *promAthPDC1:PpLUC* and 1.2 μg *35S:Renilla LUC* plasmid DNA as well as the specified amount of *35S:C2A-HRE2* plasmid. LUC activities were determined using a luminometer after 16 h of culture. Values are plotted as the average of two biological replicates, each with three technical replicates. Error bars represent $\pm$ SD.

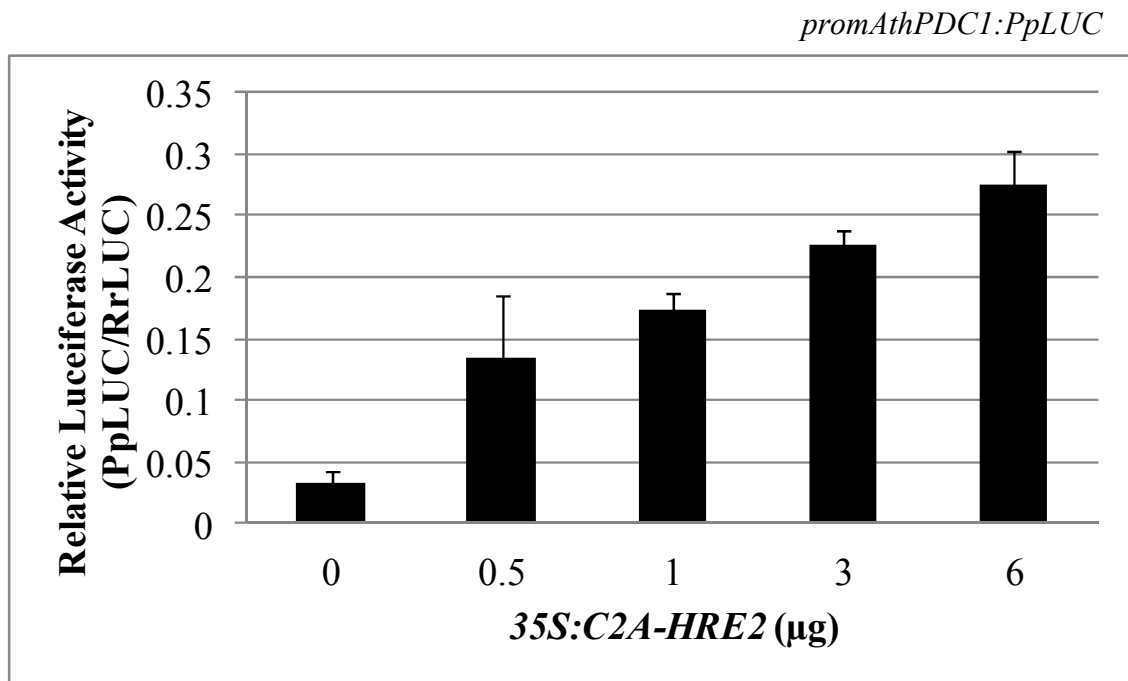


Figure 5. HRE2 activates the *Arabidopsis HRA1* promoter but its action is repressed by HRA1. Twenty-thousand *Arabidopsis* protoplasts were transfected with 4.8 μg *promAthHRA1:PpLUC*, 1.2 μg *35S:Renilla LUC*, and the amounts indicated of *35S:C2A-HRE2* and *35S:HRA1* plasmid DNA, and incubated for 20 h prior to assay of LUC activity. Values are plotted for the mean of three independent biological replicates, each with three technical replicates. Error bars represent $\pm$ SD.

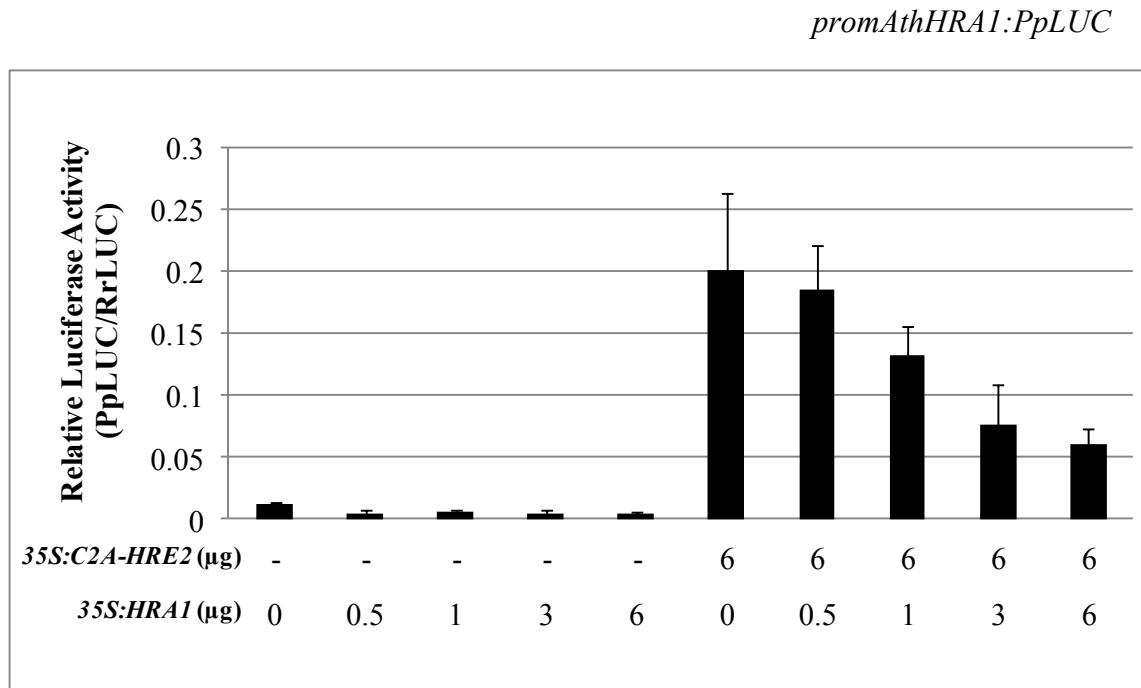
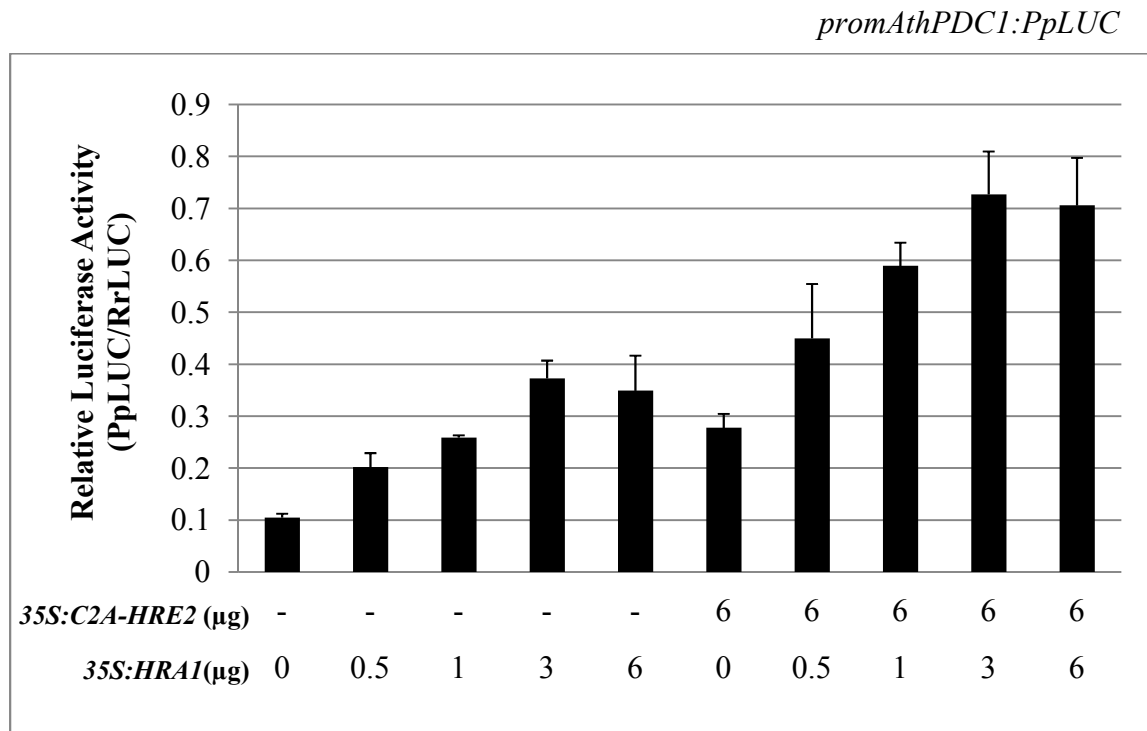


Figure 6. HRA1 activates the *Arabidopsis PDC1* promoter and promotes *C2A-HRE2* activation of the *PDC1* promoter in *Arabidopsis* protoplasts. Twenty-thousand *Arabidopsis* protoplasts were transfected with 4.8 μg *promAthPDC1:PpLUC* and 1.2 μg *35S:Renilla LUC*, in addition to the varying amounts of *35S:C2A-HRE2* and *35S:HRA1* plasmid DNA, and incubated for 20 h prior to LUC activity assay. The graph is a representation of one biological replicate, but in the other two replicates, the values for the relative luciferase activity are two and ten times lower. The luciferase activity from transactivation of *35S:HRA1* alone was also significantly lower in those experiments. Error bars represent $\pm$ SD.



3.3 RAP2.12 activates the *Arabidopsis PDC1* and *HRA1* promoters but its action is repressed by HRA1

The transcriptional activity of *35S:Δ13RAP2.12* on the *Arabidopsis PDC1* and *HRA1* promoters was investigated by a transient activation assay in *Arabidopsis* protoplasts (Figures 7 and 8). *35S:Δ13RAP2.12* drove *promAthPDC1:PP-LUC* and *promAthHRA1:PP-LUC* activity, with an increasing response at higher concentrations of the RAP2.12 plasmid. Interestingly, when *35S:Δ13RAP2.12* was co-transfected with *35S:HRA1*, activation of the *HRA1* promoter by RAP2.12 was inhibited (Figure 7). However, in contrast to the synergistic activity of *35S:C2A-HRE2* and *35S:HRA1*, it was clearly evident here that HRA1 limited the transactivation of the *PDC1* promoter by RAP2.12 (Figure 8). The antagonism between RAP2.12 and HRA1 on *PDC1* promoter activity was confirmed by a collaborator (B. Guintoli, personal communication of unpublished data). Whereas the combination of *35S:C2A-HRE2* with *35S:HRA1* displayed an upregulation of *promAthPDC1:PP-LUC* (Figure 6), a downregulation was seen with co-transfection of *35S:Δ13RAP2.12* with *35S:HRA1* (Figure 8). These results indicate that the group VII ERFs, RAP2.12 and HRE2, may have distinct functions in promoter regulation as well as interaction with HRA1.

Since RAP2.12 and HRE2 may be functionally distinct, they were co-transfected with *35S:HRA1* in assays with *promAthPDC1:PP-LUC*. Several independent experiments were performed, but one representative experiment showing the clearest distinctions in luciferase activity is illustrated in Figure 9. There was no induction of luciferase activity in the non-transfected protoplasts. Three micrograms of *35S:Δ13RAP2.12* was sufficient

to drive high transactivation of *promAthPDC1:PpLUC*. The addition of 3 µg of *35S:HRA1* repressed RAP2.12 transactivation of *PDC1* by 50%. This repression was even stronger when 6 µg of *35S:HRA1* was used. Co-transfection of *35S:Δ13RAP2.12* and *35S:C2A-HRE2* into protoplasts with *promAthPDC1:PpLUC* resulted in luciferase activity, which was maintained without the presence of *35S:HRA1*, but not as high as the RAP2.12 activation alone. The decrease in luciferase activity in a transfection of 3 µg *35S:Δ13RAP2.12* and 6 µg *35S:C2A-HRE2* suggested that HRE2 may repress RAP2.12 activity. Even lower luciferase activity was obtained when 3 µg each of *35S:Δ13RAP2.12* and *35S:C2A-HRE2* was co-transfected with 6 µg of *35S:HRA1*, supporting the idea that HRA1 acts as a negative regulator. Nonetheless, an increase in the amount of *35S:C2A-HRE2* to 6 µg restored the transcriptional activity of the *PDC1* promoter. A tenable hypothesis is that HRA1 could bind to RAP2.12 to prevent its activation of the *PDC1* promoter, but since HRE2 is not negatively regulated by HRA1, it would be HRE2 that is responsible for driving transactivation of *PDC1* when all three transcription factors are co-transfected. One caveat of this particular experiment is the absence of transfection with 3 of *35S:C2A-HRE2* alone, as this is needed side-by-side with *35S:Δ13RAP2.12* in the same transfection experiment. It would be of interest to evaluate whether this triple transfection would be different if the reporter construct was *promAthHRA1:LUC*.

Figure 7. Positive regulation of the *HRA1* promoter by *35S:AI3RAP2.12* is reversed by co-transfection with *35S:HRA1* in *Arabidopsis* protoplasts. *PromAthHRA1:PpLUC* (4.8 µg) and *35S:Renilla LUC* (1.2 µg) plasmid DNA were transfected into twenty-thousand *Arabidopsis* protoplasts, along with the amounts indicated of *35S:AI3RAP2.12* and *35S:HRA1*. The protoplasts were then incubated for 20 h prior to assay of LUC activity. Values are plotted for the average of three biological replicates, each with three technical replicates. Error bars represent $\pm$ SD.

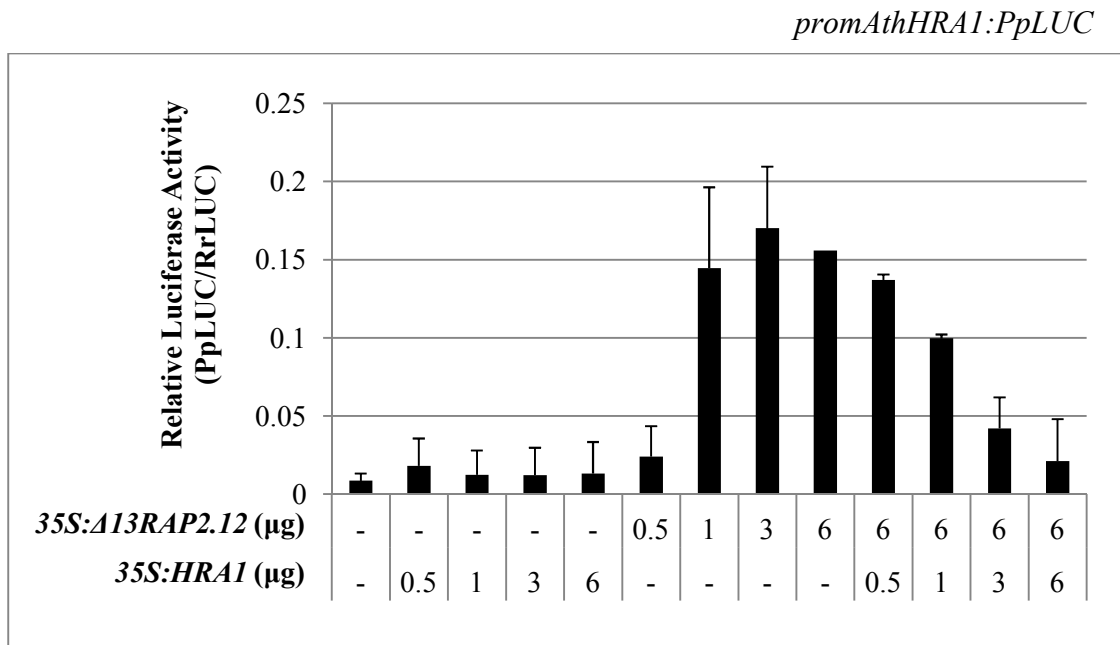


Figure 8. Positive regulation of the *PDC1* promoter by *35S:A13RAP2.12* is inhibited by *35S:HRA1* co-transfection in *Arabidopsis* protoplasts. This result is the opposite of the positive regulation observed with *35S:C2A-HRE2*. Twenty-thousand *Arabidopsis* protoplasts were transfected with *promAthPDC1:RpLUC* (4.8 μ g), *35S:Renilla LUC* (1.2 μ g), and the varying amounts indicated of *35S:C2A-HRE2* and *35S:HRA1* plasmid DNA. Protoplasts were incubated for 20 h prior to measurements of LUC activity. The graph is a representation of the average of two biological replicates, each with three technical replicates. Error bars represent $\pm$ SD.

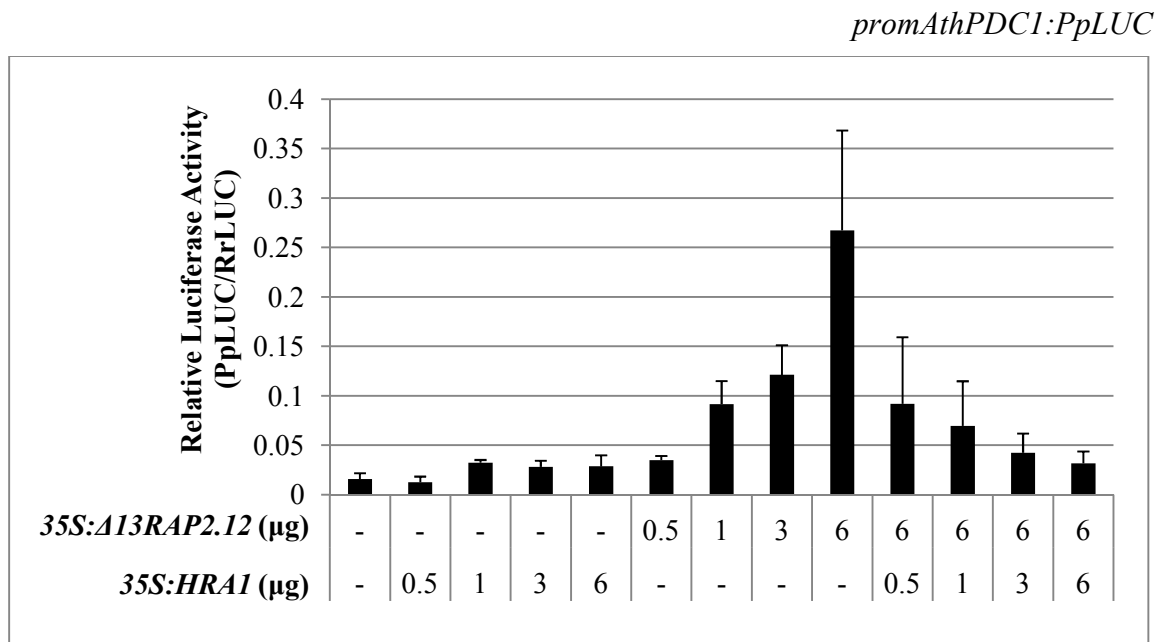
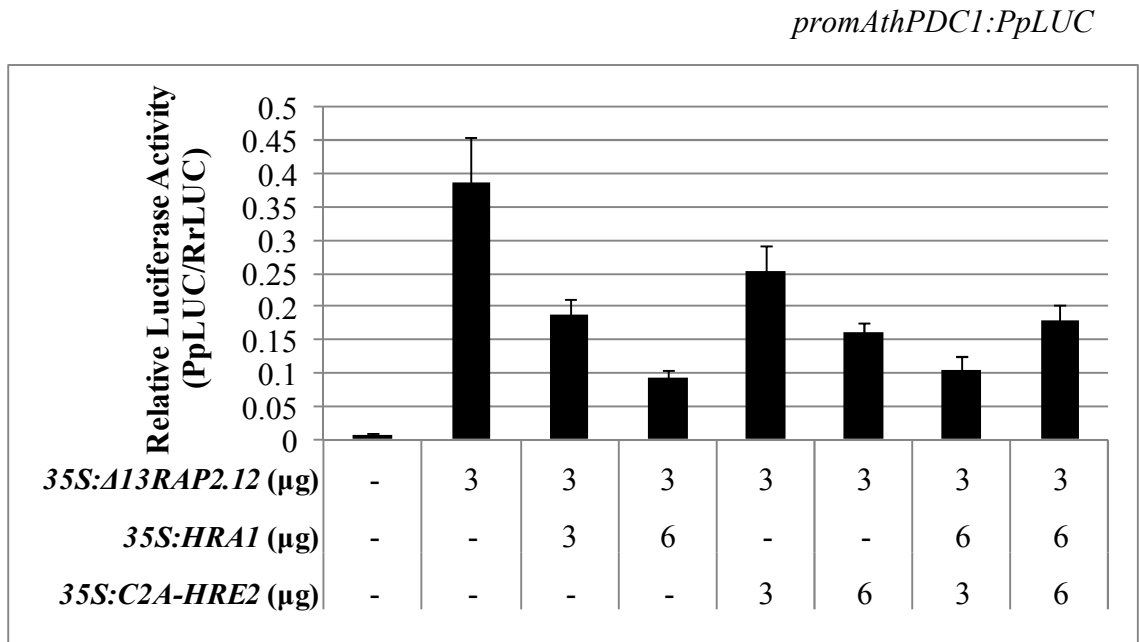


Figure 9. Co-transfection of 35S:*A13RAP2.12* with 35S:*C2A:HRE2* and 35S:*HRA1* in *Arabidopsis* protoplasts. Twenty-thousand *Arabidopsis* protoplasts were transfected with 4.8 µg *promAthPDC1:PpLUC*, 1.2 µg 35S:*Renilla LUC*, and the indicated amounts of 35S:*A13RAP2.12*, 35S:*HRA1*, or 35S:*C2A-HRE2* plasmid DNA. After a 20 h culture, LUC activity was measured. The graph is a representation of the average values of one biological replicate, but other independent experiments show similar trends. Error bars represent $\pm$ SD.



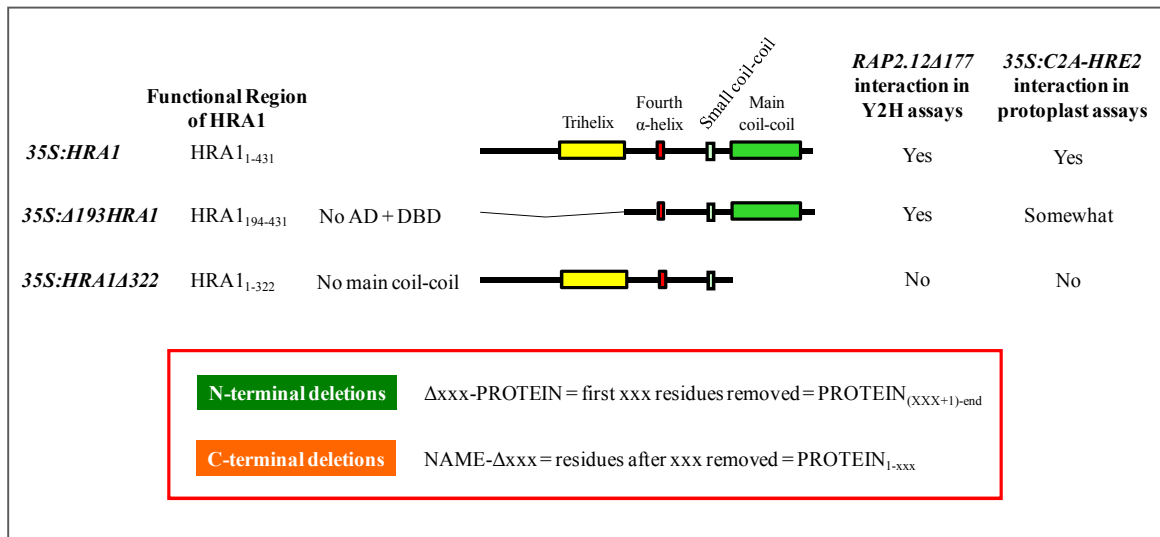
3.4 Interaction of truncated *HRA1* with *HRE2*

HRA1 encodes a trihelix transcription factor, and plays important roles in response to low oxygen. Protoplast transfection was carried out with amino (N-) and carboxyl (C)-terminal truncated *HRA1* constructs to determine whether either domain had significant biological function in regulating the promoter transactivation by group VII ERFs *RAP2.12* and *HRE2*. To accomplish this, the truncated *HRA1* regions were placed under control of the CaMV 35S promoter. *35S:HRA1Δ322* consisted of a C-terminal deletion, where amino acids after the 322nd residue were removed (Figure 10). This protein was missing the main coil-coil region, which is a structural motif consisting of two to five α -helices wrapped around each other into a left-handed helix to form a supercoil (Mason and Arndt, 2004). *35S:Δ193HRA1* had an N-terminal deletion, in which the first 193 residues were removed, thus the construct did not contain the trihelix domain, putatively functional in DNA-binding domain. These experiments sought also to confirm the interactions of the truncated *HRA1* with *RAP2.12* reported in Y2H studies (B. Guintoli, personal communication of unpublished data). *RAP2.12Δ177*, a gene construct whose region after the ERF DNA binding domain was truncated, interacted with *Δ193HRA1* but not with *HRA1Δ322* in the Y2H assays. This suggested that the C-terminal region of *RAP2.12* interacts with the C-terminal portion of *HRA1*. Y2H studies failed to show any interaction between *HRA1* and *HRE2*.

The *HRA1* deletion constructs were used in protoplast transfection assays with *35S:C2A-HRE2* (Figure 11). Low transactivation of the *HRA1* promoter by *HRE2* was

observed when transfections included the *35S:HRA1Δ322* deletion construct. The higher RAP2.12 activation ability of the *Arabidopsis HRA1* promoter, as shown in previous experiments (Figure 7), was not restored. However, transactivation of *promAthHRA1:PP-LUC* by *35S:C2A-HRE2*, in a dose-dependent manner, was confirmed in the presence of the *35S:Δ193HRA1* construct. The lower transactivation of *HRA1Δ322* can be explained by the lack of the coil-coil domain rather than the exclusion of the DNA binding domain. The results revealed consistency with the interactions of the *HRA1* truncations observed in Y2H studies of *RAP2.12Δ177*, suggesting that the same region (coil-coil domain) of *HRA1* interacts with *35S:C2A-HRE2* and *35S:RAP2.12Δ177*. Yet, this could not be proposed with complete certainty due to the low luciferase activity. The conformation of the truncated *HRA1* may have an effect on the level of luciferase induction, or post-translational modifications could be occurring in the protoplasts.

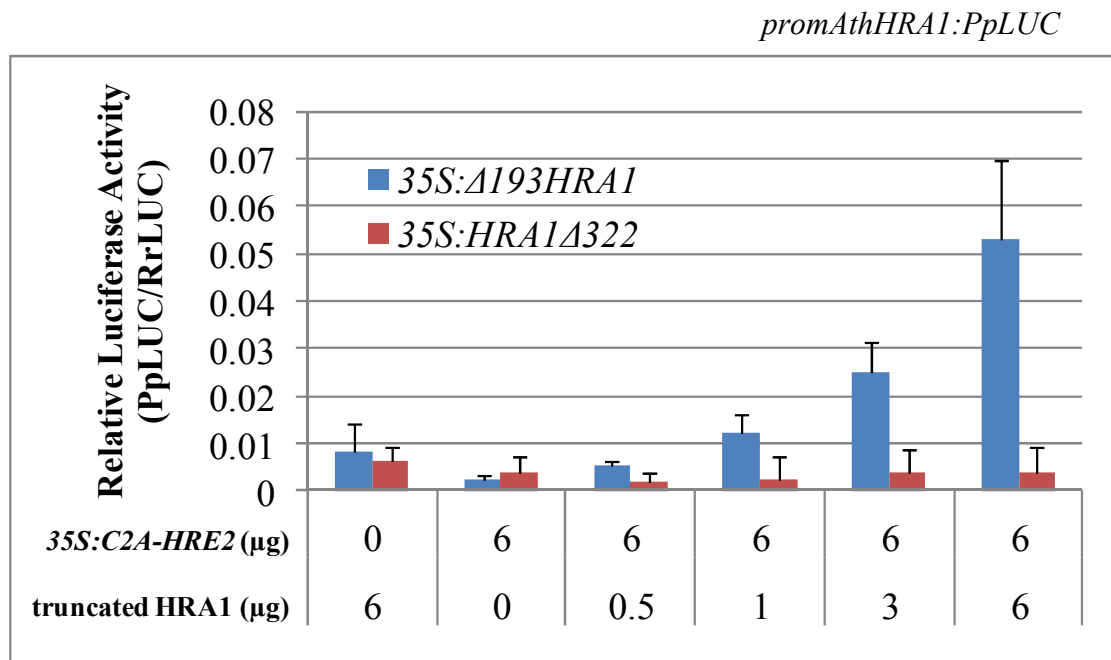
Figure 10. Illustration of the N- or C- terminal amino acid deletions in the truncated HRA1 construct, and definition of the nomenclature. Figure is adapted from B. Guintoli and S.C. Lee.



Abbreviations: AD, activation domain; DBD, DNA-binding domain.

Figure 11. Transient expression of truncated *HRA1* in *Arabidopsis* protoplasts.

35S:Δ193HRA1 drives a slight positive regulation of *35S:C2A-HRE2* under activation by *promAthHRA1:PpLUC*, while *35S:HRA1Δ322* is not functionally active. Twenty-thousand *Arabidopsis* protoplasts were transfected with *35S:C2A-HRE2* (6 μg), *promAthHRA1:PpLUC* (4.8 μg), *35S:Renilla LUC* (1.2 μg), and the amounts indicated of *35S:Δ193HRA1* or *35S:HRA1 Δ322* plasmid DNA. LUC activity was assayed after a 20 h culture. Values are shown for the average of three biological replicates, each with three technical replicates. Error bars represent ± SD.



3.5 Analysis of *SUB1A-1*, *SUB1A-2*, *SUB1C-1*, and *SUB1C-2* as transcriptional activators in rice and *Arabidopsis* protoplasts

The protoplast system was also used to identify transactivation targets of the group VII ERFs encoded at the *SUB1* locus of rice. However, it was unknown which promoter might be a target for SUB1 proteins. Moreover, the transfection of rice protoplasts proved to be considerably more challenging than that of *Arabidopsis*. These two constraints led to a systematic and incremental analysis of SUB1 protein targets, first in *Arabidopsis* protoplasts, and then in rice protoplasts.

ADHI, a gene that encodes a key anaerobic protein, was a proposed option for a promoter. Single gene studies and DNA microarray data showed that *ADHI* transcript levels were more strongly induced in the submergence tolerant rice genotypes possessing *SUB1A-1* (Fukao et al., 2006; Xu et al., 2006; Jung et al., 2010; Mustroph et al., 2012). Microarray data also revealed that *ADHI* gene was upregulated in *Arabidopsis* that ectopically expressed rice *SUB1A* and *SUB1C* (Peña-Castro et al., 2011). Based on this knowledge, it was hypothesized that *ADHI* may be targeted by *SUB1A-1*. However, transfection of rice protoplasts with *promOsADHI:PpLUC* and *35S:SUB1A-1* was inconclusive. In one bioreplicate, *35S:SUB1A-1* transactivated *promOsADHI:PpLUC* in a dose-dependent manner, but this result could not be replicated in other experiments (data not shown). When observed, the activation of *promOsADHI:PpLUC* by *35S:SUB1A-1* resulted in a very low average PpLUC/RrLUC ratio across multiple independent experiments (data not shown).

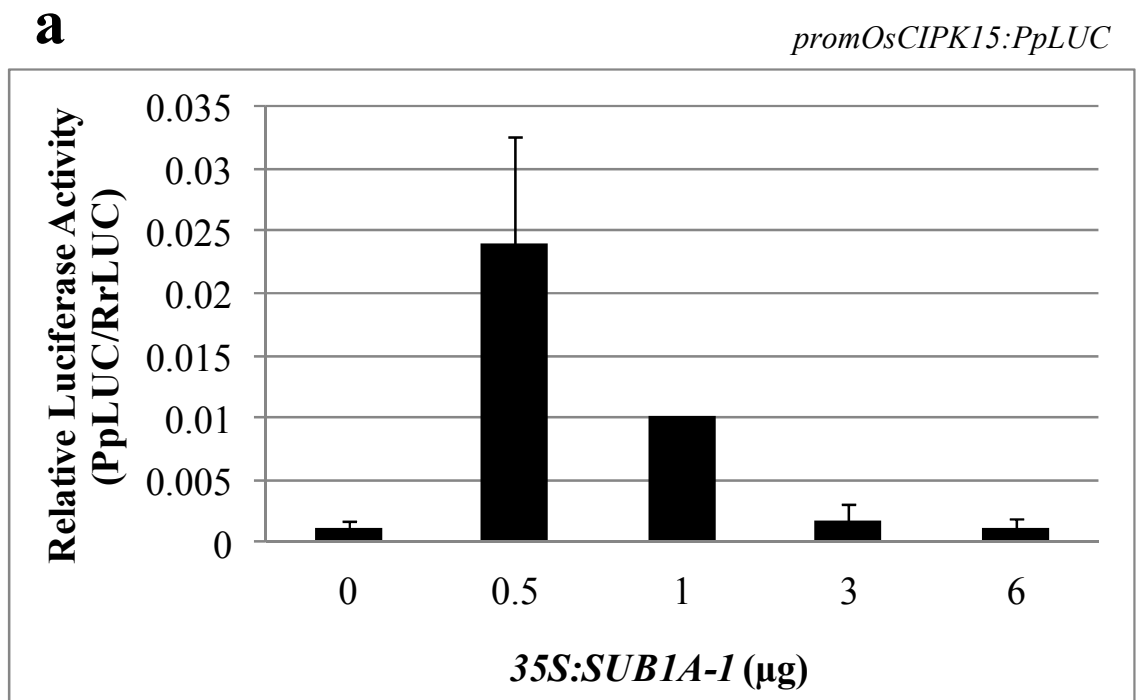
CIPK15, a key regulator of α -amylases under oxygen deprivation (Kudahettige et al., 2011), was considered another possible target of SUB1A. However, the *promOsCIPK15:PpLUC* construct was only transactivated at low but not high levels of *35S:SUB1A-1* effector plasmid (Figure 12a). Interestingly, there was a dose dependent reduction of transactivation at higher levels of *35S:SUB1A-1*. It could be possible that *SUB1A-1* was acting as an inhibitor at higher concentrations. Finally, the rice *PDC1* promoter was tested as a possible target of *SUB1*. The trend of induction and repression of activity was similar to that observed with the *CIPK15* promoter, but there was considerably higher upregulation (Figure 12b). The transactivation levels of these two promoters in rice protoplasts were both lower than observed in the *Arabidopsis* transfections.

The low upregulation of luciferase activity using the rice *ADH1*, *CIPK15*, *PDC1* promoters may indicate that these promoters are not direct targets of SUB1A, that the protein failed to accumulate in the rice protoplasts, or that the rice protoplasts were of poor quality for transfection. Despite some manipulation of the protoplasting system, the latter may be true because levels of expression of the *35S:Renilla Luciferase* was typically 45% lower in the protoplasts of rice as compared to those of *Arabidopsis*.

Since the rice promoters did not transactivate at high levels in the rice protoplasts, they were re-tested in *Arabidopsis* protoplasts using *35S:SUB1A-1* as the effector. *PromOsADH1:PpLUC* did promote luciferase expression in *Arabidopsis* protoplasts based on a single experiment, but *promAthPDC1:PpLUC* was activated at a 10-fold higher level (data not shown). With the evidence that *promAthPDC1:PpLUC* could be

transactivated by *35S:SUB1A-1* in *Arabidopsis* protoplasts, and the knowledge that co-transfection of *35S:HRA1* with a stabilized *HRE2* effector boosted *promAthPDC1:PpLUC* in *Arabidopsis* (Figure 6), the same experiment was performed in rice protoplasts using *35S:SUB1A-1* as the effector. As with the *Arabidopsis PDC1* promoter and *HRE2*, *35S:HRA1* increased the level of transactivation dramatically, as much as a 4.5-fold as compared to the *PDC1* promoter alone (Figure 13). This result may be explained by HRA1 and SUB1A working within a regulatory network in this assay.

Figure 12. *PromOsCIPK15:PpLUC* is activated by *35S:SUB1A-1* in transiently expressed rice protoplasts. *SUB1A-1* had a significant effect on *promOsCIPK15:PpLUC* (a) and *promOsPDC1:PpLUC* (b) induction with 0.5 μ g plasmid DNA, but at higher levels, there was progressive repression of transactivation. Eighty-thousand rice protoplasts were transfected with 4.8 μ g *promOsCIPK15:PpLUC* or *promOsPDC1:PpLUC*, 1.2 μ g *35S:Renilla LUC*, and the amounts indicated of *35S:SUB1A-1* plasmid DNA. Assay of LUC activity was performed after 20 h culture. Values are plotted for the mean of three technical replicates in one biological replicate. This graph is a representation of some of the other experimental replicates, but those values are significantly lower and the trend is not as apparent. Error bars represent $\pm$ SD.



b

promOsPDC1:PpLUC

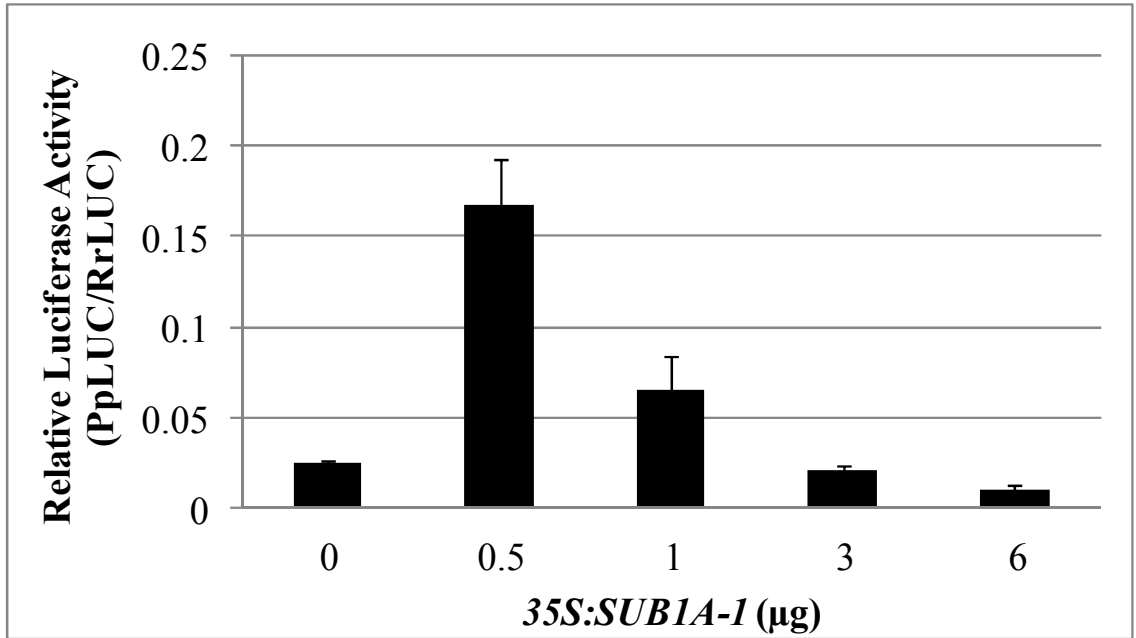
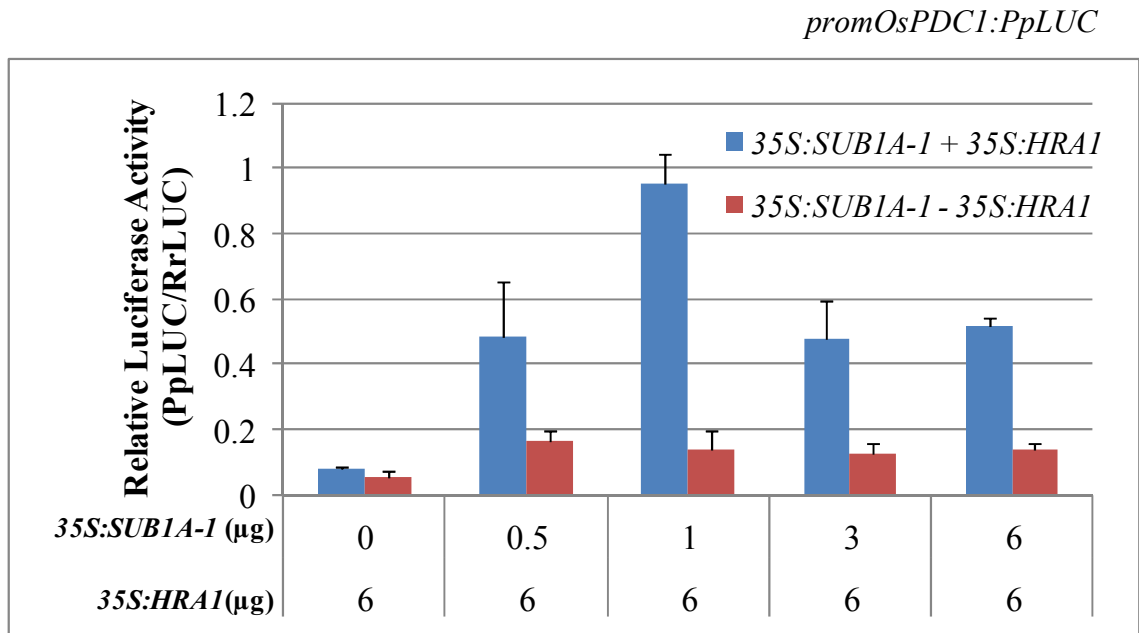


Figure 13. Transient expression of 35S:HRA1 promoting 35S:SUB1A-1 activation of *promOsPDC1:PpLUC* in rice protoplasts. The addition of 35S:HRA1 increased SUB1A-1 activity compared to 35S:SUB1A-1 alone. Eighty-thousand rice protoplasts were transfected with *promOsPDC1:PpLUC* (4.8 µg), 35S:Renilla LUC (1.2 µg), 35S:HRA1 (6 µg), and the indicated concentrations of 35S:SUB1A-1 plasmid DNA. LUC activities were assayed after 20 h of culture. Values are plotted for the mean of two biological replicates, each with three technical replicates. This graph is a representation of the trend observed in other bioreplicates, but those values are significantly lower. Error bars represent ± SD.



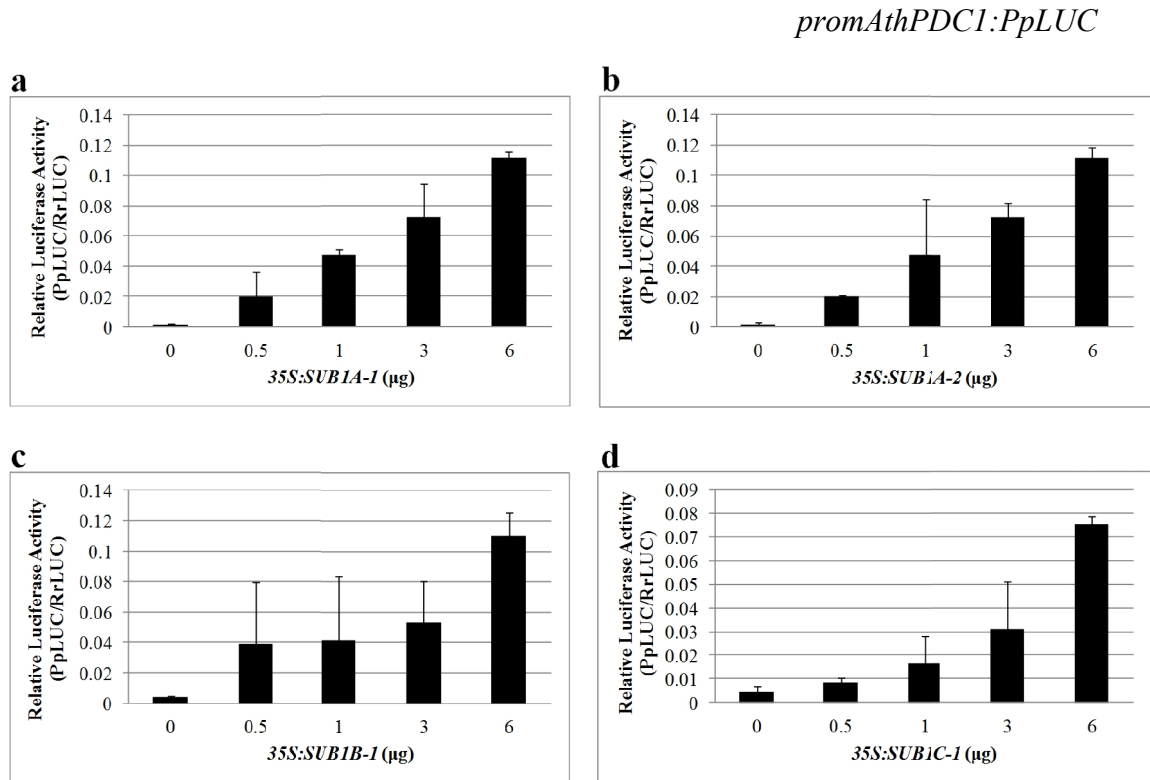
3.6 Transfection of *Arabidopsis* protoplasts to evaluate the capability of *SUB1A-1*, *SUB1A-2*, *SUB1B-1*, and *SUB1C-1* as transcriptional activators

Due to the difficulties of obtaining and transfecting rice protoplasts, further *SUB1* transfections were completed in *Arabidopsis* protoplasts. For this purpose, the *Arabidopsis PDC1* promoter was chosen to be used as the target, since preliminary experiments confirmed that *SUB1A-1* effectively transactivated this promoter. Future experiments using rice protoplasts should be performed to confirm these results.

The *Arabidopsis PDC1* promoter was used to test for transactivation by *SUB1A-1*, *SUB1A-2*, *SUB1B-1*, and *SUB1C-1* driven by the CaMV 35S promoter in *Arabidopsis* protoplasts. All *SUB1* effector constructs induced activation of *promAthPDC1:PsLUC* in a dose-dependent manner. *35S:SUB1A-1* drove *promAthPDC1:PsLUC* activity at almost a perfectly linear positive slope (Figure 14a). *35S:SUB1A-2* transactivation followed the same trend as *35S:SUB1A-1*, but with more variability at the 1 µg concentration (Figure 14b). *35S:SUB1B-1* showed strong promotion of *promAthPDC1:PsLUC* when high concentrations of plasmid were used, although a dose-dependent activation trend was still apparent (Figure 14c). *35S:SUB1C-1* also activated the *PDC1* promoter, but in a more exponential manner; the induction at 6 µg of the overexpressed *SUB1C-1* was 2.5-fold higher than at 3 µg (Figure 14d). In summary, all of the rice *SUB1 ERFs* appeared to transactivate the *PDC1* promoter, allowing a new level of resolution on the role of *SUB1* in the plant's response to submergence.

Figure 14. *35S:SUB1A-1*, *35S:SUB1A-2*, *35S:SUB1B-1*, and *35S:SUB1C-1* activates *promAthPDC1:PpLUC* when transiently expressed in *Arabidopsis* protoplasts.

Varying concentrations of *35S:SUB1A-1* (a), *35S:SUB1A-2* (b), *35S:SUB1B-1* (c), and *35S:SUB1C-1* (d) plasmid DNA were transfected into twenty-thousand *Arabidopsis* protoplasts along with 4.8 μ g *promAthPDC1:PpLUC* and 1.2 μ g *35S:Renilla LUC*. Protoplasts were incubated for 20 h prior to LUC activity assay. Values are plotted for the mean of three (a and b) or two (c and d) biological replicates, each with three technical replicates. Error bars represent $\pm$ SD.



3.7 Analysis of SAB18 as a negative regulator of *SUB1A*

SUB1A BINDING PROTEIN18 (SAB18), a rice ortholog of the trihelix protein *HRA1*, was observed in a yeast two-hybrid assay to be an interacting partner of both SUB1A and SUB1C (Seo et al., 2011). It can be hypothesized that *SAB18* functions in rice in a similar pathway to *HRA1* in *Arabidopsis*. Rice under submergence did not induce mRNA levels of SAB18 (Jung et al., 2010; Mustroph et al., 2010). Nevertheless, transient expression of the SAB18 in protoplasts might be used to obtain initial evidence of whether SAB18 functions as a regulator of submergence tolerance through *SUB1A-1* interaction.

An experiment was performed to confirm whether *SAB18* modulates the activities of its binding partner, *SUB1A*. A *35S:SAB18* construct was tested in *Arabidopsis* protoplasts using varying doses of *35S:SUB1A-1* with *promAthPDC1:PpLUC* as a reporter. However, the transactivation was low, averaging at a 0.02 firefly/Renilla ratio for luciferase activity (data not shown). The experiment was inconclusive and future studies of SAB18 are required.

3.8 Localization of GFP tagged SUB1A-1 and SUB1C-1

Determining the subcellular localization of SUB1A-1 and SUB1C-1 is valuable for a deeper understanding of the gene function. Analysis was performed using SUB1 proteins that were tagged with green fluorescent protein (GFP) and transiently expressed in *Arabidopsis*, rice, and *Nicotiana benthamiana* (tobacco) protoplast cells. Protoplasts were transfected with a *35S:GFP-SUB1A* and *35S:GFP-SUB1C* construct, with GFP fused to either the N- (p2FGW7 entry vector) or C- (p2GWF7 entry vector) terminal. *35S:GFP-*

SUB1A and *35S:GFP-SUB1C* have GFP tagged at the N-terminal of the SUB1 proteins, while *35S:SUB1A-GFP* and *35S:SUB1C- GFP* have GFP tagged at the C-terminal of the SUB1 proteins. It was hypothesized that the SUB1 proteins would accumulate in the nucleus or at the plasma membrane, based on the findings by Licausi et al. (2011) that RAP2.12 is localized at the plasma membrane under normoxic conditions. The membrane localization of SUB1s could suggest that SUB1 ERF-mediated function might be regulated by compartmentalization. However, for all of the constructs tested, GFP accumulation appeared to be confined exclusively inside the nucleus (Figures 15 and 16). DAPI staining identified the nuclei of the protoplasts. As a control, untransformed protoplast cells yielded no GFP expression.

The strongest GFP signal was detected using tobacco (*Nicotiana benthamiana*) protoplasts (Figure 15a and 15b), in which GFP signals appeared to be confined to the nucleus. *SUB1A-1* and *SUB1C-1* were also nuclear-localized in *Arabidopsis* protoplasts (Figure 15c and 15d). Some C-terminally GFP-tagged protoplasts did not accumulate GFP in the nucleus as strongly as in the N-terminally GFP-tagged protoplasts. It is likely that the nuclear targeting sequence is at the N-terminal of *SUB1A-1* and *SUB1C-1* since that region contains a number of basic residues typical of a nuclear localization signal (Xu et al., 2006; Fukao et al., 2009).

There were more difficulties detecting the GFP signal in rice protoplasts, possibly due to the low transformation efficiency (Figure 16). The GFP signals appeared to be localized to the nuclei of protoplasts, but the GFP accumulation was not as strong as observed in *Arabidopsis* or *N. benthamiana* protoplasts. A higher signal was not even

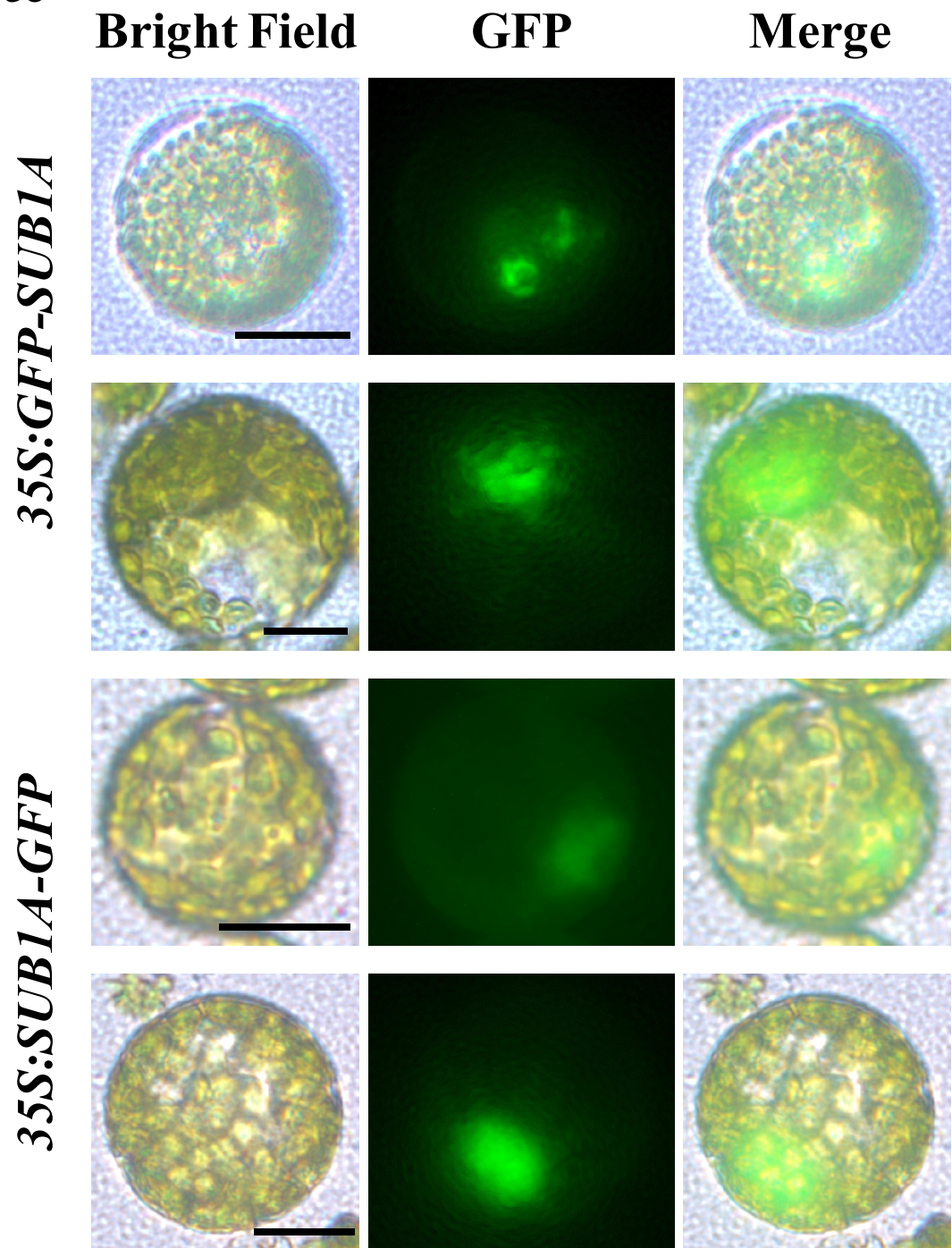
observed when the N-terminus was tagged with the GFP. The SUB1 proteins may not have been completely stable in the rice protoplasts. Reducing oxygen levels may have been necessary for stronger accumulation of the protein. Future experiments would involve a time course of expression, as the signal may be brighter after a shorter culture period.

Based on these results, it appears that *SUB1A-1* and *SUB1C-1* are exclusively or at least primarily nuclear-localized. However, there must be further confirmation using other approaches, such as by fractionation of the protoplasts into cytoplasmic and nuclear fractions, which are then subjected to immunoblot detection using an anti-GFP antibody. Additional control studies using RAP2.12, with and without the removal of N-terminal sequences associated with N-end rule degradation, would have been appropriate. These are important as we had anticipated from the results with C-terminally tagged RAP2.12 (Licausi et al., 2011), that these proteins might be associated with the plasma membrane.

Figure 15. Subcellular localization of N- and C- terminal GFP fusion of *SUB1A-1* and *SUB1C-1* transiently expressed in *N. benthamiana* and *Arabidopsis* protoplasts.

Twenty-thousand *N. benthamiana* (a, b) or *Arabidopsis* protoplasts (c, d) were transfected with 4.8 µg *promOsPDC1:GFP*, 1.2 µg *35S:Renilla LUC*, and 3 µg of the N- or C- terminally GFP-tagged *SUB1A* or *SUB1C* plasmid DNA. After 20 h of culture, pictures were taken using bright-field and GFP filters. Images were obtained with a Leica DM LB2 light microscope. The scale bars represent 20 µm for *N. benthamiana* cells and 10 µm for *Arabidopsis* cells.

a



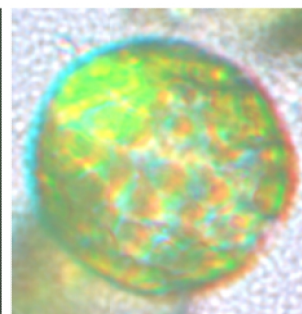
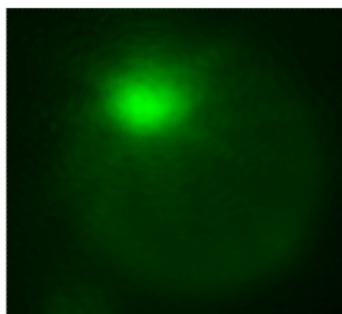
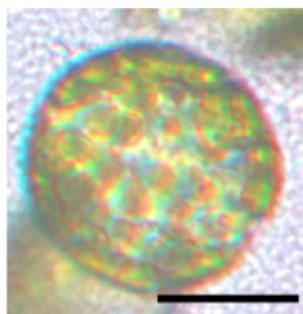
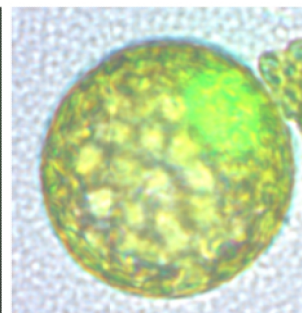
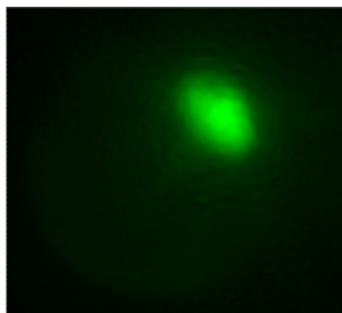
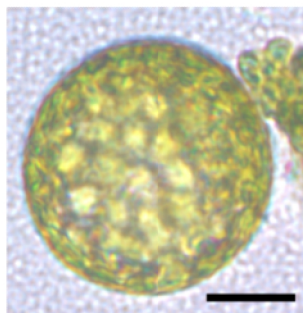
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35S:GFP-SUB1C

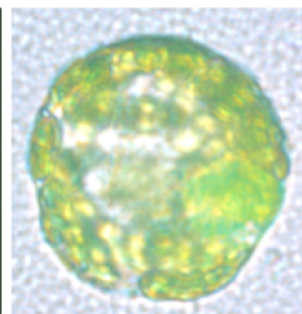
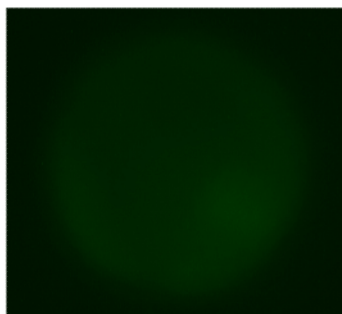
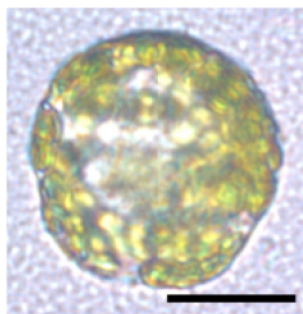
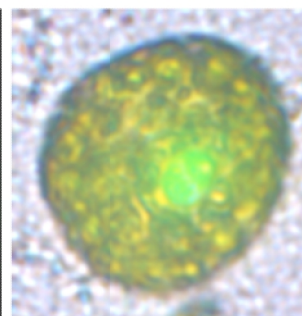
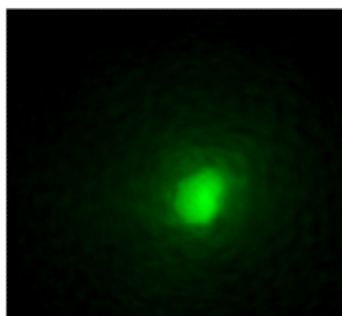
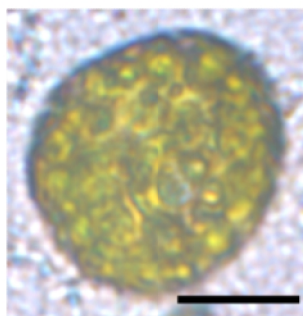
Bright Field

GFP

Merge



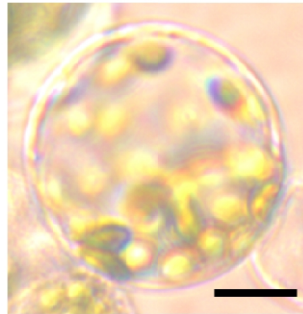
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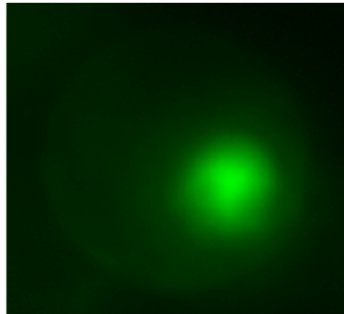
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35S::GFP-SUB1A

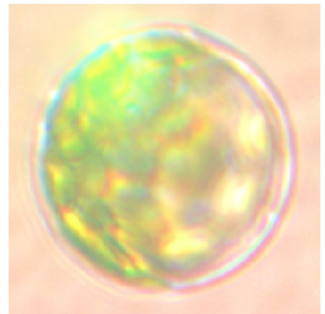
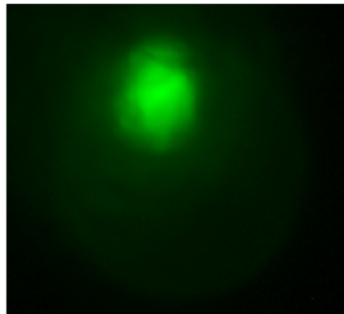
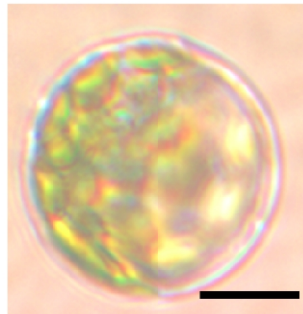
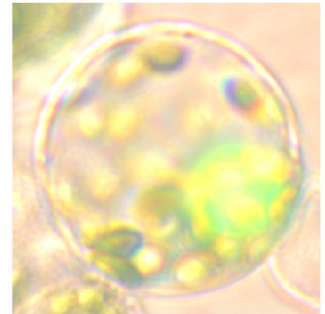
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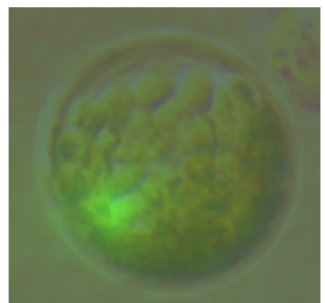
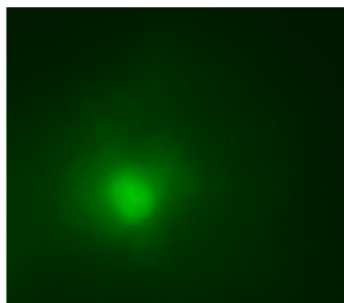
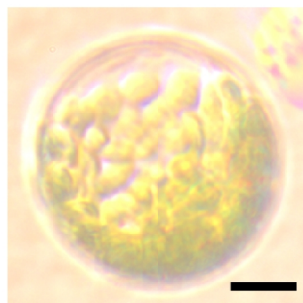
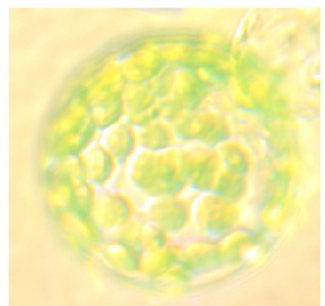
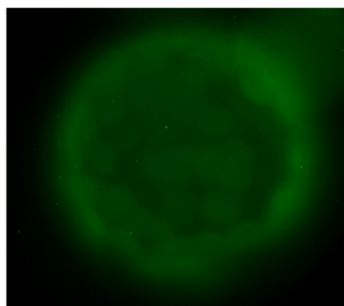
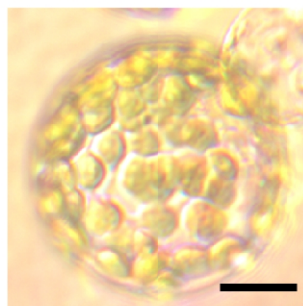
GFP



Merge



35S::SUB1A-GFP



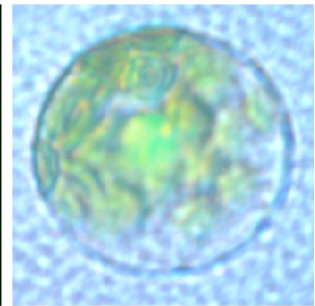
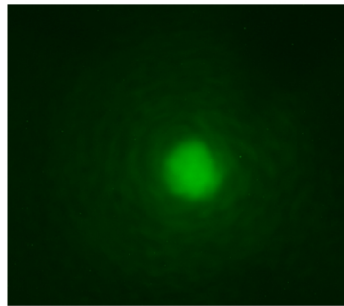
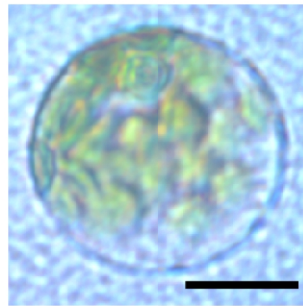
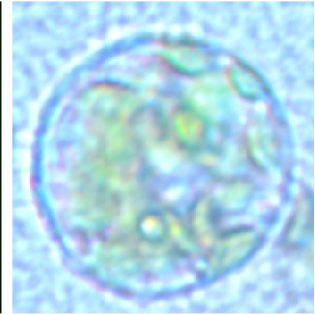
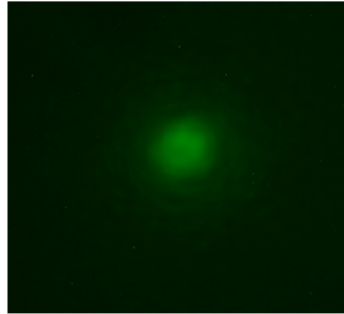
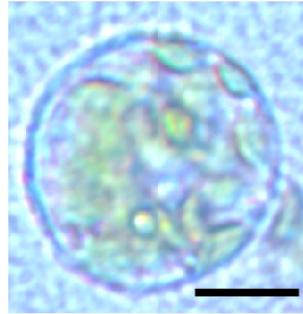
d

Bright Field

GFP

Merge

35S:GFP-SUB1C



35S:SUB1C-GFP

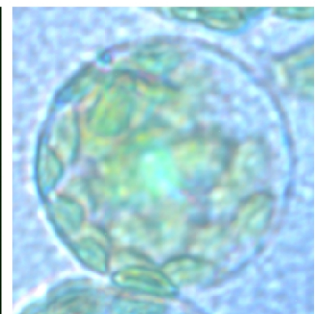
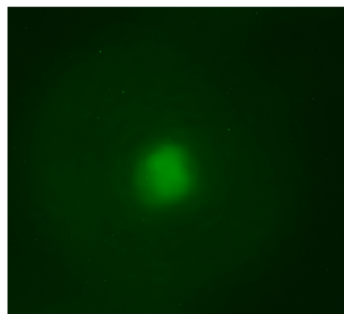
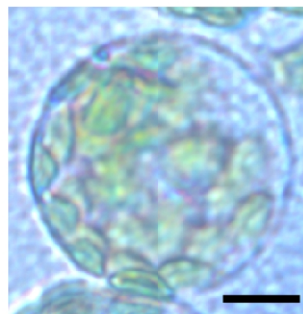
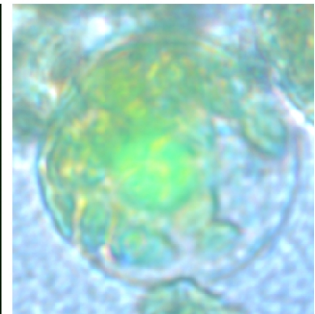
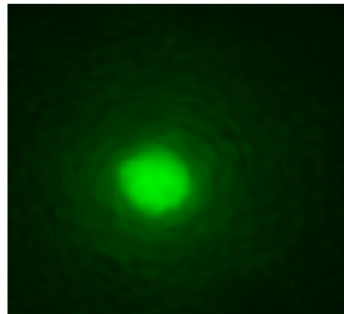
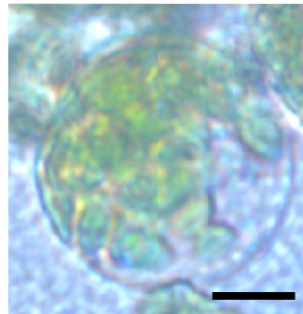
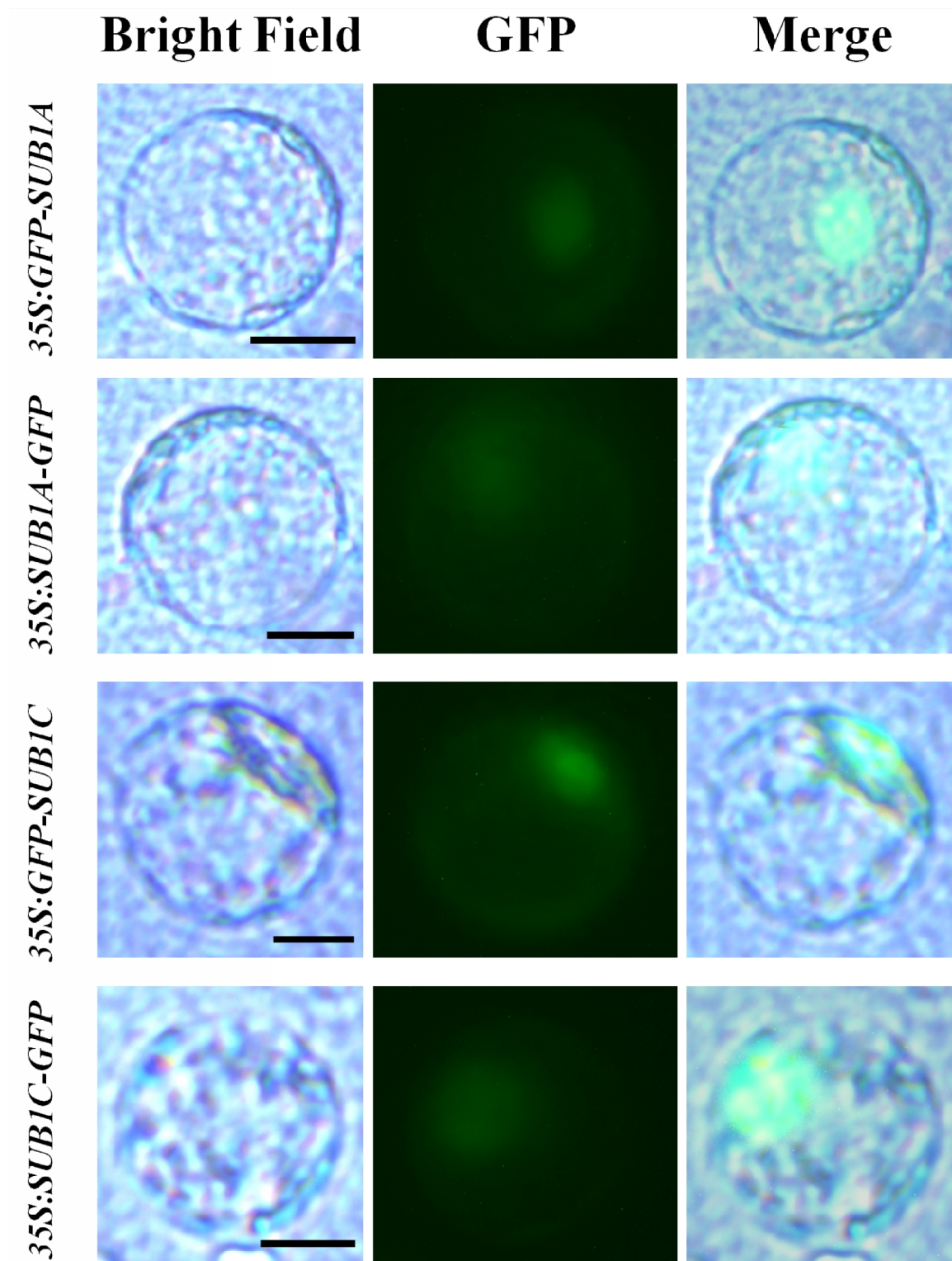


Figure 16. Subcellular localization of N- and C- terminal GFP fusion of *SUB1A-1* and *SUB1C-1* transiently expressed in rice protoplast. Approximately eighty-thousand rice protoplasts were transfected with 4.8 μg *promOsPDC1:PpLUC*, 1.2 μg *35S:Renilla LUC*, and 3 μg of the N- or C- terminally GFP-tagged *SUB1A* or *SUB1C* plasmid DNA. Protoplasts were viewed under a Leica DM LB2 light microscope after 20 h of culture using bright-field and GFP filters. The scale bars represent 5 μm .

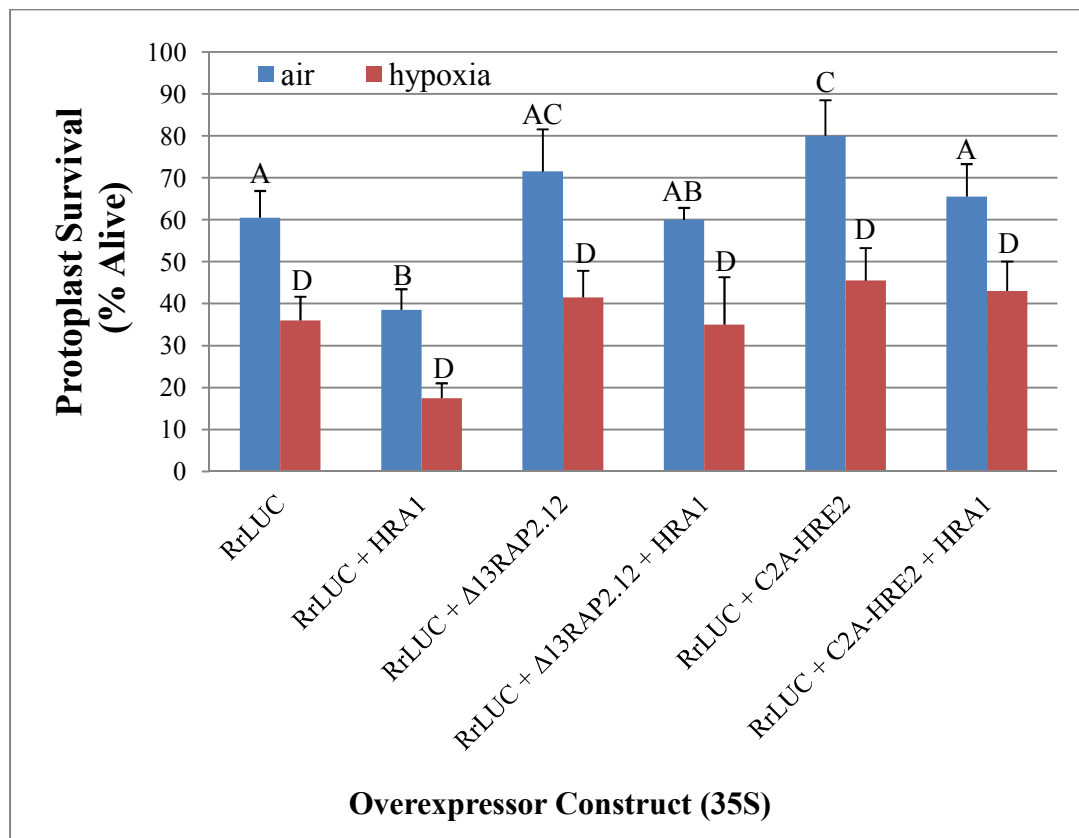


3.9 Measure of protoplast viability using 35S:*RrLUC*

The viability of the protoplasts was determined by staining with Evan's blue dye, in which dead cells are stained blue due to an inability to exclude the dye. The protoplasts were observed under a light microscope and the ratio of viable versus non-viable protoplasts was calculated after counting cells with a hemacytometer. Healthy protoplasts were round whereas stressed or dying cells often appeared irregular or lumpy. After the enzymatic digestion, *Arabidopsis* protoplasts were released with nearly 85–90% viability. Reassessment was performed again after transfection of the protoplasts and a 20 h culture period.

An experiment to measure viability was performed using 35S:*RrLUC* and other constructs to determine transfection efficiency and survival under two culture conditions: humidified air under light (typical incubation conditions) and humidified Argon in the dark (hypoxic conditions) (Figure 17). Our collaborator observed that 35S:*Δ13RAP2.12* had a positive effect on protoplast viability in the air (B. Guintoli, unpublished data), but this could not be replicated due to the high variability between bioreplicates. Yet, this assay confirmed that the increase in protoplast survivability was abolished by co-transfection with HRA1. 35S:*C2A-HRE2* conferred slightly higher protoplast survivability, as observed with 35S:*Δ13RAP2.12*. Viability of the protoplasts similarly dropped when 35S:*HRA1* was co-transfected with 35S:*C2A-HRE2*. The survivability declined across all transfections for protoplasts incubated under a hypoxic condition, and there were no significant difference among the samples. The 20 h incubation under hypoxia may be too extensive, so this might be tested with a shorter incubation period.

Figure 17. Measure of *Arabidopsis* protoplast survivability using 35S:*RrLUC* and overexpressed *HRA1*, *C2A-HRE2*, Δ 13RAP2.12 constructs. Twenty-thousand *Arabidopsis* protoplasts were transfected with 0.6 μ g 35S:*Renilla LUC* and 3 μ g of the effector plasmid DNA. The number of viable versus dead protoplasts was scored using the vital stain Evan's blue after a 20 h transfection in humidified air or hypoxic conditions. Error bars represent $\pm$ SD between two independent replicates. Means with different letters above bars indicate significant difference from the control and from each other based on ANOVA and Tukey HSD analysis ($p < 0.05$).



Chapter 4

DISCUSSION

Transient protoplast transfections can characterize functional roles of genes

Transient gene expression assays in protoplasts, using promoter of genes fused to luciferase, seek to understand how a gene is regulated. This can be regulation that is controlled by development or a response to a particular environmental condition, such as hypoxia or submergence (Yoo et al., 2007). The basis of the observed luciferase activity is dependent on fusing a promoter region and 5' untranslated region upstream of a gene to a luciferase coding sequence. Since *cis*-acting enhancer elements are expected to be in the cloned promoter region, the promoter-luciferase activity observed with the dual luciferase reporter assay is an indicator of the promoter activity. Transcriptional activation can be mediated by factors within the protoplast or by a factor driven by a constitutive promoter that is delivered to the protoplast at the same time as the promoter-luciferase construct. In this way, transcription factors that act on specific promoters or *cis*-acting sequences within the plant can be easily investigated. Although the assays do not provide direct evidence of an effector binding to the reporter's promoter, they can indicate either direct or indirect activation. These assays provide information that can be validated by another method. This study presented here illustrated how the protoplast transactivation system is a useful method for characterizing group VII ERFs signaling interactions related to low oxygen and submergence responses in *Arabidopsis* and rice.

Transactivation experiments of RAP2.12, HRE2, and HRA1 reveal distinctions of relevance between various group VII ERFs

The *Arabidopsis* protoplast transfection experiments showed that stabilized versions of RAP2.12 and HRE2 transactivate the *HRA1* and *PDC1* promoters in a dose-dependent response (Figures 3, 4, and 8), indicating that both group VII ERFs, although distinctly regulated in the plant, can regulate these two hypoxia-responsive genes. Furthermore, the data presented here provide the insight that the trihelix protein HRA1 affects transactivation mediated by both RAP2.12 and HRE2 but in a distinct manner (Figure 18). Although HRA1 did not appear to transactivate either the *HRA1* or *PDC1* promoter on its own, it negatively regulated the transcriptional activation capability of RAP2.12 on these promoters (Figures 7 and 8). The effects of co-transfection of *HRA1* with *HRE2* were more complex. There was repression of transactivation of the *HRA1* promoter when *35S:C2A-HRE2* and *35S:HRA1* were co-transfected (Figure 5), as observed with the dual transformation RAP2.12 and HRA1 constructs. However, HRE2's transactivation of the *PDC1* promoter was not a target of negative regulation by HRA1 (Figure 6). Instead, co-transfection of HRE2 and HRA1 constructs had a synergistic positive effect on the transactivation of *promAthPDC1:PgLUC*.

The contrasting effect of HRA1 on RAP2.12 and HRE2 regulation of the *PDC1* promoter indicates that these two ERFs either target the promoter in a distinct manner or are differently regulated. Based on unpublished Y2H data by our collaborator (B. Guintoli, personal communication), the ERF and the coil-coil region of the trihelix factor physically interact. If this is assumed to be correct, then the core hypoxia-responsive

genes include a negative regulator of the targets of RAP2.12 (i.e., the core hypoxia responsive genes *PDC1* and *HRA1*). In fact, Lee (2012) demonstrated that overexpression of *HRA1* significantly dampens the upregulation of many hypoxia responsive mRNAs, including *PDC1* and *HRE2*. Physical interaction between RAP2.12 and HRA1 could rapidly inhibit the transcriptional response to the stress.

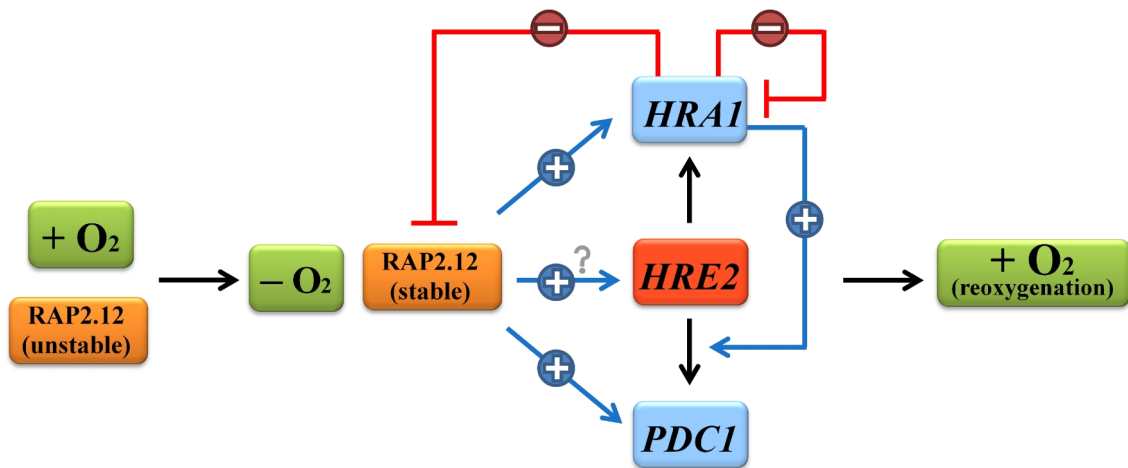
To explain the promoter-specific action of HRA1 on HRE2 transactivation we will postulate that the two proteins also interact. This is supported by the preliminary demonstration that the HRA1 coiled-coil domain co-transfected with HRE2 was sufficient to enhance HRE2 transactivation of the *PDC1* promoter (Figure 11). Several scenarios can be proposed. HRA1 could promote HRE2 transactivation of *PDC1* and not *HRA1* due to distinctions in HRA1 interaction with the two promoters or other transcription factors involved in their activation. For example, HRA1 could bind to the *HRA1* promoter but not the *PDC1* promoter. A more indirect mechanism is also possible, in which HRA1 drives expression of another factor that promotes HRE2 transactivity on the *PDC1* promoter. Beyond these interactions, HRE2 could contribute to a feedback loop that acts on HRA1 to downregulate its repressive effects, although this would have to be promoter specific because HRE2 and HRA1 together negatively regulated the *HRA1* promoter.

The results involving co-transfection of RAP2.12 and HRE2 further suggest that HRA1 can promote HRE2 activity. There was an inhibition of RAP2.12 activation of *promAthPDC1:PpLUC* with 3 and 6 µg of 35S:*HRA1*, but when RAP2.12 was co-transfected with HRE2, some activation ability was restored (Figure 9). There was some

repression of transactivation when HRA1 was added to the co-transfection, but when the dosage of HRA1 was increased there was an increase of ERF activity on the target *PDC1* promoter. This likely reflects the synergistic HRA1 and HRE2 activation of the *PDC1* promoter. A possibility is that HRA1 could sequester RAP2.12 away from the system by rendering the transcriptional activation capability of RAP2.12 inactive, thus allowing HRE2 to freely activate the *PDC1* promoter. Future investigations to resolve these possibilities should include targeted protein-protein interaction studies as well as validation of the factors binding to specific *cis*-elements of these promoters.

Figure 18 summarizes the dissection of the activity of RAP2.12, HRE2, and HRA1 on regulation of the *PDC1* and *HRA1* promoters tested using the protoplast assay. These results uncover positive and negative regulation of two core hypoxia responsive genes. The proposal that RAP2.12 alone promotes HRE2 expression is still hypothetical since it has not been tested.

Figure 18. Proposed model describing the overlapping regulation of HRA1 on RAP2.12 and HRE2 under low oxygen stress in *Arabidopsis*. The hypothesized model was revised based on the results obtained. Red and blue lines indicate the interactions tested. Positive and negative regulations are depicted by “plus” and “minus” signs, respectively. The relationship that RAP2.12 promotes HRE2 expression is hypothetical since it was not tested.



Transformation difficulties with rice protoplasts

Protoplast isolation and transformations were performed in both *Arabidopsis* and rice systems. The published protocol to obtain and transfect the *Arabidopsis* protoplasts was easily established, but isolation of rice protoplasts required extensive troubleshooting. The stem was the main tissue source for rice protoplast isolation, as it provided the highest yield of protoplasts compared to only using leaf tissue or using leaf and stem tissue combined. However, the amount of rice protoplasts obtained was comparatively lower to that of leaf-derived *Arabidopsis* protoplasts. Stem and sheath tissue were sliced from approximately 100 rice plants compared to approximately 25 leaves cut in *Arabidopsis*, but the yield of protoplasts in rice was as much as three times lower. It may require more than 300 rice plants for each experiment to obtain enough protoplasts for transfection. It is possible that the amount of cellulase in the extraction buffer may need to be increased since some cell wall structures were not broken down when the protoplast suspension was observed under a microscope. A slightly higher mannitol concentration was used for osmotic stabilization in the rice protoplasts, but other modifications to the protoplast extraction solution and the buffer may be required. In addition, the rice protoplasts were smaller in size compared to *Arabidopsis* protoplasts, which may also have affected the transfection efficiency.

In some experiments, the rice protoplasts obtained were not viable when tested with Evan's blue dye staining. In the first few experiments for isolation of rice protoplasts, the 6 to 8 h incubation period may have been too extensive compared to a 3 h incubation time for *Arabidopsis* protoplasts. To determine the optimal incubation time

required for maximal release of the rice protoplasts, aliquots of protoplasts were counted using a hemocytometer every hour up to 8 h. It appeared that a 4.5 h incubation was sufficient to obtain the maximal amount of viable protoplasts.

Despite numerous transfection experiments, the rice protoplasts did not display high transactivation of the rice *ADHI*, *CIPK15*, or *PDC1* promoter-luciferase constructs. *35S:SUB1A-1* appeared to drive the *ADHI* promoter in one experiment, but the results were difficult to reproduce in the rice protoplasts. It is possible that other genes may be more suitable targets for *SUB1* activity, but it is more likely that low transfection efficiency resulted in lower detection of the luciferase activity. The induction of the *ADHI* promoter could also be an artifact of overexpression of the protein or the inadvertent hypoxia stress. In addition, the rice protoplasts potentially could be less viable after transfection with certain constructs, as co-transfection of combinations of *35S:Δ13RAP2.12*, *35S:C2A-HRE2*, *35S:HRA1*, and *35S:RrLUC* affected the viability of *Arabidopsis* protoplasts (Figure 17). Intriguingly, *35S:HRA1* strongly induced *PDC1* promoter activity (Figure 13). It is possible that *SUB1A-1* may be a target of HRA1, a concept that can contribute to the homologous network of the group VII ERFs. However, more studies will need to be performed to confirm this proposal.

Transfection of *SUB1* in *Arabidopsis* protoplasts drives the *Arabidopsis* *PDC1* promoter

Transfection of *Arabidopsis* with the rice *SUB1* gene constructs provided convincing evidence that *SUB1A-1*, *SUB1A-2*, *SUB1C-1*, and *SUB1C-2* were capable of

transactivation of *promAtPDC1*. This suggests that all four ERFs may have the capability to bind the same *cis*-element, presumably also targeted by RAP2.12 and HRE2 in the protoplast assay. A promoter deletion analysis followed by site-directed mutagenesis of the target could lead to the identification of this element. The *Arabidopsis PDC1* promoter contains a few known ERF binding motifs, including the ATCTA motif related to hypoxic responses (Welsch et al., 2007; Licausi et al., 2011) and GCC box (AGCCGCC) that is present in the promoter regions of many ethylene-inducible genes (Hinz et al., 2010; Jung et al., 2010). Further experiments are needed to carefully determine the involvement of specific ERF binding motifs in regulating the activity of the *Arabidopsis PDC1* promoter.

The trends of the promotion or repression of relative luciferase activity were intriguingly different between rice and *Arabidopsis* protoplasts. In *Arabidopsis* protoplasts, *SUB1A-1*, *SUB1A-2*, *SUB1C-1*, and *SUB1C-2* drove positive transactivation under increasing dosage of the effector plasmid (Figures 14). Yet, in rice protoplasts, there was high induction of luciferase activity at a 0.5 µg concentration, but the activity dropped significantly at 1 µg, and even further at 3 and 6 µg (Figures 12). This could be explained by different means of regulation (i.e., other interacting proteins or post-translational modifications) between the two plant species. The results might also be interpreted as evidence that at high levels these ERFs have a repressive function. The data obtained from *SUB1* gene transfection in *Arabidopsis* protoplasts would have been more informative and practical using rice protoplasts, but this will not be possible until the transfection efficiency of the rice protoplasts is optimized.

In summary, this study assessed the binding of group VII ERF transcription factors to *cis*-regulatory elements using an approach involving PEG-mediated transformation in *Arabidopsis* and rice protoplast cells. The luciferase gene fusions provided insight into the mechanisms that regulate the activity of specific genes in plants under hypoxic or submergence stress. Transactivation assays revealed that most group VII ERFs (*SUB1A-1*, *SUB1A-2*, *SUB1B-1*, *SUB1C-1*, and *RAP2.12*) positively regulate core hypoxia responsive genes in both *Arabidopsis* and rice, but further research would be necessary to answer the questions raised by the results presented here, especially in regards to the more complicated regulations involving HRE2 and HRA1. This study provided a preliminary understanding of the molecular mechanisms that govern plants' enhanced response to low oxygen and submergence stress.

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