Two Alternative Splicing Variants of AtERF73/HRE1, HRE1α and HRE1β, Have Differential Transactivation Activities in Arabidopsis

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Abstract: AtERF73/HRE1 is an AP2/ERF transcription factor in Arabidopsis and has two distinct alternative splicing variants, HRE1α and HRE1β. In this study, we examined the differences between the molecular functions of HRE1α and HRE1β. We found that HRE1α and HRE1β are both involved in hypoxia response and root development and have transactivation activity. Two conserved motifs in the C-terminal region of HRE1α and HRE1β, EELL and LWSY-like, contributed to their transactivation activity, specifically the four E residues in the EELL motif and the MGLWS amino acid sequence at the end of the LWSY-like motif. The N-terminal region of HRE1β also showed transactivation activity, mediated by the VDDG motif, whereas that of HRE1α did not. The transactivation activity of HRE1β was stronger than that of HRE1α in Arabidopsis protoplasts. Both transcription factors transactivated downstream genes via the GCC box. RNA-sequencing analysis further supported that both HRE1α and HRE1β might regulate gene expression associated with the hypoxia stress response, although they may transactivate different subsets of genes in downstream pathways. Our results, together with previous studies, suggested that HRE1α and HRE1β differentially transactivate downstream genes in hypoxia response and root development in Arabidopsis.

Keywords: alternative splicing; Arabidopsis; HRE1; hypoxia; root development; transactivation

1. Introduction

To survive under stressful conditions, plants change the expression patterns of stress-responsive genes. In this process, transcription factors are the trans-acting elements that play major roles in regulating gene expression by binding to cis-acting elements [1]. The APETAL2/ethylene-responsive factor (AP2/ERF) transcription factor family consists of transcription factors that are mainly plant-specific but are also found in protists, cyanobacteria, and phages [2]. Members of the AP2/ERF superfamily are encoded by 145 loci in Arabidopsis and 167 loci in rice [3–5]. AP2/ERF transcription factors are divided into four subfamilies: AP2, ERF, dehydration-responsive element-binding factor/C-repeat-binding factor (DREB/CBF), and RAV [4,6]. They function as either transcriptional activators or repressors in gene regulation. The specific conserved activation domains of these activators are not well-known, except for the LWRY motif, which has been identified at the C-terminus of ERFs and DREB/CBFs [7–11]. Acidic amino acid residues, such as aspartate (D) and glutamate (E), participate in the transactivation activity of these activators [12]. In contrast, repressors have distinct repressor domains, such as the ERF-associated...
amphiphilic repression motif, TLLLFR motif, and B3 repression domain [13–15]. Transcription factors in the ERF subfamily bind to the GCC box (5′-AGCGGCC-3′) and/or to dehydration-responsive element/C-repeat (DRE/CRT) (5′-A GCCGAC-3′) to regulate the expression of downstream genes [16,17].

The splicing of pre-mRNA is a crucial step in the expression of the information encoded in eukaryotic genomes [18]. Alternative splicing occurs when splice sites are differentially recognized; more than one transcript and multiple potential proteins are generated from the same pre-mRNA [18]. The splice sites that are selected under particular cellular conditions are determined by the interactions of proteins, globally designated as splicing factors, which guide spliceosomal components and subsequently the spliceosome to the respective splice sites [19–21].

Abiotic stresses, such as heat, cold, salinity, and drought, markedly change alternative splicing patterns, thus implementing changes in gene expression as a part of the adaptive responses of plants to adverse environments. Splicing factors themselves also change their expression or activity under stress conditions [22]: distinctive splicing variants are expressed in different tissues or undergo degradation via nonsense-mediated decay [22]. Splicing variants may also vary with regard to their subcellular localization or have different biological functions; for example, HfsA2 generates a truncated protein by alternative splicing under heat stress and acts as a positive regulator of its own transcription [23]; bZIP60, encoding a key transcription factor in the unfolded protein response, generates a nucleus-targeted alternative splicing variant under heat stress, while it generates an endoplasmic reticulum-localized protein under normal conditions. The splicing variant of bZIP60 localized in the nucleus activates the transcription of genes regulating protein folding and degradation [24,25]. In contrast, ZIFL1 generates two alternative splicing variants encoding full-length and truncated proteins localized in the tonoplast of root cells and the plasma membrane of leaf stomatal guard cells, respectively; the full-length protein regulates auxin transport in the root cells, while the truncated protein mediates drought tolerance in the stomatal guard cells [26]. Although the regulation and functions of abiotic stress alternative splicing variants have been studied in plants, their role and downstream pathways in hypoxia responses have not yet been investigated, except in a small number of high-throughput studies [27,28]. Hypoxia is a growth-limiting factor, especially for non-oxygen-evolving organs or green tissues under dark conditions [29–31], leading to a decrease in ATP production and a subsequent energy crisis affecting numerous plant processes. To circumvent a negative energy status, plants have developed several strategies, including a switch to anaerobic fermentation to regenerate the NAD\textsuperscript{+} required to sustain glycolytic ATP production [29,30].

AtERF73/HRE1 (HRE1) is an AP2/ERF transcription factor in Arabidopsis that belongs to group VII of the ERF subfamily and contains one AP2/ERF domain [32]. HRE1 has two distinct alternative splicing variants, HRE1α and HRE1β [33], whose overexpression confers tolerance to flooding or anoxia [33,34]. Moreover, HRE1α-overexpressing transgenic plants (OXs) exhibit increased primary root elongation due to elevated root cell division [33]. It has been also reported that HRE1α has transactivation activity, supposedly imputable to its C-terminal region [33]. Although previous studies have examined each splicing variant, no study has yet compared HRE1α and HRE1β, nor their transactivation activity, the domains responsible for their activity, and the downstream genes that they affect.

In this study, we assessed the functional differences between HRE1α and HRE1β. Expression pattern analyses suggested that HRE1β plays a more important role than HRE1α in the hypoxia response, and analysis of HRE1α OXs and HRE1β OXs showed that both splicing variants are involved in the hypoxia response and root development. Our results demonstrated that both HRE1α and HRE1β have two motifs in their C-terminal region that contribute to their transactivation activity. HRE1β has an additional motif in its N-terminal region that is also involved in its transactivation activity. RNA-sequencing (RNA-Seq) analysis of genes downstream of HRE1α and HRE1β revealed that both these splicing variants might function differentially as transcriptional activators via the GCC box in Arabidopsis.
2. Results

2.1. Structural Comparison of HRE1α and HRE1β

Comparison of the genomic structures of HRE1α and HRE1β showed that HRE1α does not contain any intron, whereas HRE1β does have one (Figure 1a), and HRE1α and HRE1β encode proteins that potentially have 211 and 262 amino acids (aa), respectively (Figure 1b). HRE1α and HRE1β share 200 amino acids, including the AP2/ERF domain and C-terminal region, whereas the N-terminal region of HRE1β is longer than that of HRE1α because it harbors N-degron pathway-targeted sequences (Figure 1b) [35]. We performed BLASTP analysis to identify orthologs of both HRE1α and HRE1β in other plant species. Interestingly, no orthologs of HRE1α contained sequences similar to those in its N-terminal region. In contrast, all the orthologs corresponded to HRE1β, containing N-degron pathway-targeted sequences in their N-terminal regions (Figure 1c) [35]. In addition, HRE1α and HRE1β have no paralogs in Arabidopsis, whereas some of the other orthologs have paralogs in the same species (Figure 1c). We generated a phylogenetic tree to compare the phylogenetic relationship among HRE1α, HRE1β, and its orthologs. HRE1α and HRE1β were the two most closely related proteins and were close to the orthologs in Camelina sativa and Capsella rubella (Figure 1d).

**Figure 1.** Splicing variant isoforms of HRE1. (a) The genomic structures of HRE1α and HRE1β. Black box, gray box, and line indicate coding region, untranslated region, and intron, respectively. (b) The protein domains of HRE1α and HRE1β. Dark gray box, light gray box, and white box indicate the common region of HRE1α and HRE1β, HRE1α-specific N-terminal region, and HRE1β-specific N-terminal region, respectively. Boxes with deviant crease line indicate the AP2/ERF domain. (c) Multiple alignment
among HRE1α, HRE1β, and the orthologs of HRE1β in other plant species. Multiple alignment was performed with the amino acid sequences of the full-length ORFs of HRE1α, HRE1β, and the orthologs of HRE1β using Clustal Omega. (d) Phylogenetic tree of HRE1α, HRE1β, and the orthologs of HRE1β was generated with the full-length ORF. WP_096680639, an AP2 domain-containing protein from Fischerella sp. NIES-4109 was used as an outgroup.

2.2. Comparison of HRE1α and HRE1β Functions in the Hypoxia Response and Root Development

The expression of both HRE1α and HRE1β is upregulated by hypoxia [33,34]; to compare their expression patterns in response to such stress, the transcript levels of both splicing variants were analyzed under hypoxia conditions. Ten-day-old wild-type (WT) seedlings treated under low-oxygen conditions were analyzed by quantitative reverse-transcription PCR (RT-PCR) using HRE1α or HRE1β-specific primers (Table S1). As a result, the expression of both HRE1α and HRE1β was found to be higher after the hypoxia treatment, which was consistent with previous results (Figure 2a and Figure S1) [33,34]. The transcript levels of HRE1β were significantly higher than those of HRE1α under hypoxic conditions (Figure 2a).

Next, we examined the hypoxia responses of HRE1α OX and HRE1β OX seedlings. For this, HRE1β OXs were generated and selected using quantitative RT-PCR (Figure S2). The HRE1α OXs generated in previous study were used [33]. HRE1α OX, HRE1β OX, and WT seeds were sown onto the same plate and treated with hypoxia 7 days after germination (DAG). After 18 or 24 h of hypoxia treatment, both HRE1α OX and HRE1β OX seedlings were more tolerant than WT, with higher survival percentages (Figure 2b,c); we also found no significant phenotypic difference between the HRE1α OXs and HRE1β OXs (Figure 1b,c). Despite a previous report stating that hre1 mutants do not show significant differences from the WT under hypoxia conditions [34], these results suggested that both HRE1α and HRE1β are involved in the hypoxia response.

In a previous study, we showed that HRE1α is involved in primary root elongation via regulation of root meristem cell division [33]. In addition, the expression level of HRE1β has been shown to be higher in the roots than in the shoots [34]. To compare the functions of HRE1α and HRE1β in root development, we analyzed their expression in the shoots and roots using 7- and 14-day-old WT seedlings. The expression levels were similar in the shoots of both 7- and 14-day-old seedlings (Figure 2d). However, the expression level of HRE1α was significantly higher than that of HRE1β in the roots (Figure 2d).

We next compared the root lengths of the HRE1α and HRE1β OXs. For this, the HRE1α and HRE1β OX seeds were sown onto the same Murashige and Skoog (MS) plates as the WT seeds and the primary root lengths of the seedlings were measured at 7 DAG. Both of the OXs had longer primary roots than the WT plants (Figure 2e,f), and the primary root length did not significantly differ between the HRE1α OXs and HRE1β OXs (Figure 2e,f). These results suggest that both splicing variants function in primary root development. However, hre1 mutants did not show significant difference in root elongation compared to WT (data not shown).
of -D-galactopyranoside (ONPG) assay and yeast growth assay, C91 showed the highest activity among was thus investigated in this study using the yeast system. As a result, we found that the full-length transactivation activity, although it was weaker than that of C91 (Figure 3c,d). However, M109, the three regions, almost the same as that of the full-length HRE1 responsible for this activity [33]. However, the same was not previously investigated for HRE1 2.3. N-Terminal and C-Terminal Regions of HRE1α and HRE1β Are Responsible for Transactivation Activity

Previously, we reported that HRE1α has transactivation activity and that its C-terminal region is responsible for this activity [33]. However, the same was not previously investigated for HRE1β and was thus investigated in this study using the yeast system. As a result, we found that the full-length HRE1β showed transactivation activity in yeast, which was very similar to that of the full-length HRE1α (Figure 3c,d and Figure S3a). These results indicated that both HRE1β and HRE1α function as transcriptional activators.

To identify the domain responsible for its transactivation activity, HRE1β was divided into three regions: the N-terminal 1–73-aa region (N73), middle 63–171-aa region (M109), and C-terminal 172–262-aa region (C91) (Figure 3b). In the quantitative β-galactosidase 2-nitrophenyl-β-D-galactopyranoside (ONPG) assay and yeast growth assay, C91 showed the highest activity among the three regions, almost the same as that of the full-length HRE1β (Figure 3c,d). N73 also showed transactivation activity, although it was weaker than that of C91 (Figure 3c,d). However, M109, containing the AP2/ERF DNA-binding domain, did not show transactivation activity (Figure 3c,d). These results indicated that both the N-terminal and C-terminal regions of HRE1β contain domains with transactivation activity, unlike HRE1α, in which only the C-terminal region shows activity.
2.4. N-Terminal Region of HRE1\(\beta\) Has One Motif Responsible for Transactivation Activity

We identified the orthologs of HRE1\(\beta\) N73 in other plant species, including *Arabidopsis lyrata*, *Camelina sativa*, *Capsella rubella*, *Eutrema salsugineum*, *Brassica napus*, and *Brassica oleracea*, using the BLASTP tool. Multiple alignment among HRE1\(\beta\) N73 and its orthologs was performed using the Clustal Omega tool. Forty-seven amino acids in HRE1\(\beta\) N73, corresponding to the 1–47-aa region in HRE1\(\beta\), were highly conserved among HRE1\(\beta\) and its orthologs (Figure 4a). The first 10 amino acids in this sequence, MCGGAVISDY, are known to be related to the N-degron pathway, which is involved in the protein degradation process [35].

To identify the motif(s) responsible for transactivation activity in HRE1\(\beta\) N73, partial fragments of HRE1\(\beta\) N73, namely, N73-N (1–47 aa), N73-C (48–73 aa), and N73-\(\Delta\)10 (11–47 aa; N73-N without 1–10 aa), were fused to GAL4 DNA-binding domain (BD) (Figure 4b). In the quantitative \(\beta\)-galactosidase ONPG assay and yeast growth assay, both N73-N and N73-\(\Delta\)10 showed transactivation activity, while N73-C did not (Figure 4c,d). In addition, there was no significant difference in the activity between N73-N and N73-\(\Delta\)10 (Figure 4c,d), indicating that the 37-aa sequence of N73-\(\Delta\)10 is important for transactivation activity. We named the highly conserved 26-aa sequence in the N73-\(\Delta\)10 (22–47 aa) as the VDDG motif (Figure 4a).

![Figure 3. Analysis of transactivation activities of HRE1\(\alpha\) and HRE1\(\beta\).](image-url)
HRE1β, and truncated fragments of HRE1β for the analysis of transactivation activity in yeast. The transactivation activities were quantified by measuring the β-galactosidase activity in yeast extract. The experiments of each technical replicate were performed in triplicate. The data shown are the means ± S.D. (n = 3). Similar results were obtained from at least three biological replicates, with one shown here. The data were analyzed with one-way ANOVA using Tukey’s multiple comparison test. Different letters indicate significant differences (p < 0.05). (d) Yeast growth assay. Yeast transformants were grown on SM-Trp/-Ura. In (c,d), pBD-GAL4 vector itself was used as a negative control.

2.5. C-Terminal Region of HRE1β Has Two Motifs Responsible for Transactivation Activity

We next analyzed the motif(s) in the C-terminal region of HRE1α/HRE1β responsible for transactivation activity. First, we compared the activity of HRE1β C91 (172–262 aa) with that of HRE1β C81 (182–262 aa) because the 121–130-aa region of HRE1α corresponding to the 172–181-aa region of HRE1β has been shown to be unimportant for the activity [33]. Quantitative β-galactosidase ONPG assay revealed that both C91 and C81 show transactivation activity, with no significant difference between the activities of these two regions (Figure 5c,d).

We further analyzed the motif(s) responsible for transactivation activity in HRE1β C81. BLASTP analysis using HRE1β C81 identified orthologs in other plant species, such as Arabidopsis lyrata, Camelina sativa, Capsella rubella, Eutrema salsugineum, Raphanus sativus, Brassica napus, and Brassica rapa, and multiple alignment between them was performed (Figure 5a). Based on the conservation of amino acid sequences, HRE1β C81 was divided into two subregions: HRE1β C81-N (182–213 aa) and HRE1β C81-C (214–262 aa) (Figure 5b). The results of quantitative β-galactosidase ONPG and yeast growth assays showed that both C81-N and C81-C exhibited transactivation activity, and the activity of C81-N was lower than that of C81-C (Figure 5c,d).

To narrow the possible motif(s) responsible for transactivation activity, HRE1β C81-C was divided into two subregions: C81-C-1 (214–238 aa) and C81-C-2 (239–262 aa) (Figure 5b). The results of quantitative β-galactosidase ONPG assay and yeast growth assay showed that both C81-C-1 and C81-C-2 exhibited transactivation activity, with that of C81-C-1 being higher than that of C81-C-2 (Figure 5c,d). These results suggested that both the C81-C-1 and C81-C-2 contain motif(s) are responsible for transactivation activity.
Four E residues (E220, E221, E226, and E230) in HRE1β C81-C-1 were highly conserved among HRE1β and its orthologs (Figure 5a). To assess their importance for the transactivation activity of C81-C-1, each E residue was replaced with an alanine (A) residue by point mutation, resulting in mC81-C-1-1 (E220A E221A), mC81-C-1-2 (E226A E230A), and mC81-C-1-3 (E220A E221A E226A E230A) (Figure 6a). Quantitative β-galactosidase ONPG and yeast growth assays showed that mC81-C-1-1 and mC81-C-1-2 showed similar activity to the nonmutated C81-C-1, whereas mC81-C-1-3 showed almost no activity (Figure 6b,c). These results indicated that all four E residues (E220, E221, E226, and E230) in the C-terminal region of HRE1β are necessary for its transactivation activity.

The LWSY motif of RAP2.12 and RAP2.2 in Arabidopsis, which belong to group VII of the ERF subfamily, is important for the transactivation activity of these transcription factors [8]. HRE1β C81-C-2 also has amino acid sequences similar to the LWSY motif at its C-terminal end; the sequences were found to be highly conserved among HRE1β and its orthologs (Figure 5a). To analyze whether LWSY-like sequences are important for transactivation activity, C81-C-2 without LWSY-like sequences (C81-C-2ΔLWS) was fused to GAL4 BD (Figure 6d). The results of quantitative β-galactosidase ONPG and yeast growth assays showed that C81-C-2ΔLWS had significantly lower activity than C81-C-2 (Figure 6e,f), suggesting that LWSY-like sequences consisting of five amino acids in HRE1β C81-C-2...
contribute to the transactivation activity. We named the 21-aa sequence of C81-C-1 (218–238 aa) as the EELL motif and the five amino acids of C81-C-2 (MGLWS) as the LWSY-like motif (Figure 5a).

**Figure 6.** Identification of the amino acid residues responsible for transactivation activity. (a) The amino acid sequences of HRE1β C81-C-1 and its site-directed mutated sequences for the analysis of transactivation activity in yeast. (b,e) Quantitative β-galactosidase ONPG assay. The transactivation activities were quantified by measuring the β-galactosidase activity in yeast extract. The experiments of each technical replicate were performed in triplicate. The data shown are the means ± S.D. (n = 3). Similar results were obtained from at least three biological replicates, with one shown here. The data were analyzed with one-way ANOVA using Tukey’s multiple comparison test. Different letters indicate significant differences (p < 0.05). (c,f) Yeast growth assay. Yeast transformants were grown on SM-Trp/-Ura. (d) The schematic maps of HRE1β C81-C-2 and its partial fragment for the analysis of transactivation activity in yeast. The numbers in parentheses represent the position in HRE1α. In (b,c,e,f), pBD-GAL4 vector itself was used as a negative control.

2.7. HRE1α and HRE1β Show Transactivation Activity In Vivo

Transactivation assay systems including effector, reporter, and reference plasmids, have been widely used to compare the transactivation activities of proteins and/or their domains in plant protoplasts [36–38]. To confirm the transactivation activity of HRE1α and HRE1β in *Arabidopsis*, effector plasmids, in which each full-length open reading frame (ORF) of HRE1α and HRE1β was fused to GAL4 BD, were generated and transformed into *Arabidopsis* protoplasts (Figure 7a). Transient expression of the effector plasmid of HRE1α or HRE1β with the reporter plasmid, harboring the firefly luciferase gene under the control of 4X GAL4 upstream activating sequence, showed that the full-length ORFs of both HRE1α and HRE1β exhibit transactivation activity in *Arabidopsis* protoplasts (Figure 7b). The activity of HRE1β was found to be higher than that of HRE1α in *Arabidopsis* protoplasts (Figure 7b).

The transactivation activity of HRE1β N73, HRE1β M109, and HRE1β C81 was also assessed in *Arabidopsis* protoplasts. HRE1β C81 showed strong activity in *Arabidopsis* protoplasts, while HRE1β N73 showed weak activity, and HRE1β M109 did not (Figure 7b). These results were similar to those found in yeast (Figure 3).
Figure 7. Analysis of transactivation activities of HRE1α and HRE1β. (a) The schematic maps of the effector plasmid, reporter plasmid, and reference plasmid for the analysis of transactivation activities of full-length ORFs of HRE1α and HRE1β, and truncated fragments of HRE1β. (b) The relative firefly luciferase activity in *Arabidopsis* protoplasts. The transformation efficiency was normalized with Nano luciferase activity of the reference plasmid. The normalized firefly luciferase activity of negative control was set as 1. The empty effector plasmid was used as a negative control. The data shown are the means ± S.D. (n = 3). The data were analyzed with one-way ANOVA using Tukey’s multiple comparison test. Different letters indicate significant differences (p < 0.05).

2.8. Both HRE1α and HRE1β Transactivate Downstream Genes via the GCC Box

It was previously shown that HRE1α transactivates downstream genes via the GCC box [33]. To investigate whether HRE1β also regulates downstream genes via the GCC box, effector plasmids for the expression of HRE1α and HRE1β were transformed into *Arabidopsis* protoplasts with reporter plasmids containing the firefly luciferase gene under the control of 4× GCC box core sequences (Figure 8a). Transactivation assay results showed a great relative increase of reporter activity by both HRE1α and HRE1β (Figure 8b), suggesting that both transcription factors transactivate downstream genes via the GCC box. Recently, it was reported that hypoxia-responsive promoter element (HRPE) is a *cis*-acting element regulated by members from group VII of the ERF subfamily, including *HRE1, HRE2, RAP2.12, RAP2.2, and RAP2.3* [39]. However, we did not ascertain whether HRE1α and HRE1β transactivate downstream genes via HRPE because HRE1 (HRE1β) does not bind to HRPE [39].
were downregulated in HRE1α OXs and HRE1β OXs, respectively (Figure 9a). Among these, 26 genes were upregulated in both the HRE1α OXs and HRE1β OXs (Figure 9a). In contrast, 69 and 282 genes were downregulated in HRE1α OXs and HRE1β OXs, respectively (Figure 9b). Among these, 32 genes were downregulated in both the HRE1α OXs and HRE1β OXs (Figure 9b). The distribution of these differentially regulated genes is shown using volcano plots (Figure 9c,d). These results indicated that HRE1α and HRE1β could differentially transactivate downstream genes, and HRE1β could transactivate more genes than HRE1α.

To validate the RNA-Seq results, we analyzed the expression of the genes upregulated in HRE1α OXs and/or HRE1β OXs. First, we confirmed that the expression of HRE1α and HRE1β was upregulated in the HRE1α OXs and HRE1β OXs, respectively (Figure 10a,b). We selected the upregulated genes, namely, WRKY46, BBX16/EIP6, At2g46670, ASG4, AGP30, and BBE8, in HRE1α OXs and/or HRE1β OXs from the RNA-Seq analysis results (Tables S3 and S4) for use in expression analysis. The results showed that WRKY46 and BBX16/EIP6, which were upregulated in both the HRE1α OXs and HRE1β OXs, had higher expression levels in both the HRE1α OXs and HRE1β OXs than in the WT plants.

Figure 8. Transactivation assay of HRE1α and HRE1β via GCC box. (a) The schematic maps of the effector plasmid, reporter plasmid, and reference plasmid for the transactivation assay of HRE1α and HRE1β via GCC box. (b) The relative firefly luciferase activity in Arabidopsis protoplasts. The transformation efficiency was normalized with Nano luciferase activity of the reference plasmid. The normalized firefly luciferase activity of negative control was set as 1. The empty effector plasmid was used as a negative control. The data shown are means ± S.D. (n = 3). The data were analyzed with one-way ANOVA using Tukey’s multiple comparison test. Different letters indicate significant differences (p < 0.05).
Expression levels of At2g46670 and ASG4, which were upregulated only in HRE1α OXs, were confirmed to be higher in HRE1α OXs than in WT plants; no significant difference in their expression was detected between HRE1β OXs and WT plants (Figure 10a). The expression levels of AGP30 and BBE8, which were upregulated in HRE1β OXs, were higher in HRE1β OXs than in WT plants; no significant difference in the expression levels of these genes was detected between HRE1α OXs and WT plants (Figure 10g,h). These results were consistent with the RNA-Seq results.

**Figure 9.** Differentially expressed genes in HRE1α OXs and/or HRE1β OXs. (a) The numbers of genes that were upregulated in HRE1α OXs and/or HRE1β OXs relative to those in WT plants with >2-fold differences in expression and a p < 0.05. (b) The numbers of genes that were downregulated in HRE1α OXs and/or HRE1β OXs relative to those in WT plants with >2-fold differences in expression and a p < 0.05. (c) Volcano plot of DEGs identified between WT and HRE1α OXs. (d) Volcano plot of DEGs identified between WT and HRE1β OXs. In (c) and (d), DEGs were selected by p < 0.05 and log2 ratio ≥ 1 conditions.
The genes upregulated in both HRE1α and HRE1β in HRE1α OXs and HRE1β OXs. These results suggested that HRE1α and HRE1β might transactivate genes in different downstream pathways. The genes upregulated in both HRE1α OXs and HRE1β OXs were enriched in biological processes involved in response to abiotic stimulus, response to light stimulus, response to endogenous stimulus, response to oxygen-containing compound, and response to lipid (Tables 1 and 2), indicating that both HRE1α and HRE1β might be involved in abiotic stress responses.

**Table 1.** List of GO terms of genes upregulated more than 2-fold in HRE1α OXs.

| GO Term                  | Description                           | Number in Input List | p-Value          |
|--------------------------|---------------------------------------|----------------------|------------------|
| GO:0048511               | Rhythmic process                      | 5                    | 1.14 × 10^{-3}   |
| GO:0009909               | Regulation of flower development      | 5                    | 1.85 × 10^{-3}   |
| GO:2000241               | Regulation of reproductive process    | 6                    | 1.90 × 10^{-3}   |
| GO:0009628               | Response to abiotic stimulus          | 16                   | 2.54 × 10^{-3}   |
| GO:0009416               | Response to light stimulus            | 9                    | 3.08 × 10^{-3}   |
| GO:0009314               | Response to radiation                 | 9                    | 3.98 × 10^{-3}   |
| GO:0048831               | Regulation of shoot system development| 5                    | 4.85 × 10^{-3}   |
| GO:0009639               | Response to red or far red light      | 5                    | 6.66 × 10^{-3}   |
| GO:0007623               | Circadian rhythm                      | 4                    | 7.67 × 10^{-3}   |
| GO:0009719               | Response to endogenous stimulus       | 13                   | 1.46 × 10^{-2}   |
| GO:0009739               | Response to gibberellin               | 4                    | 1.49 × 10^{-2}   |
| GO:0010468               | Regulation of gene expression         | 18                   | 1.73 × 10^{-2}   |
| GO:0048580               | Regulation of postembryonic development| 5                    | 2.43 × 10^{-2}   |
Table 1. Cont.

| GO Term                   | Description                                               | Number in Input List | p-Value       |
|---------------------------|------------------------------------------------------------|----------------------|---------------|
| GO:0048367                | Shoot system development                                   | 8                    | 2.94 × 10^{-2}|
| GO:0010017                | Red or far-red light signaling pathway                    | 3                    | 3.13 × 10^{-2}|
| GO:0051171                | Regulation of nitrogen compound metabolic process          | 17                   | 3.21 × 10^{-2}|
| GO:0071489                | Cellular response to red or far red light                 | 3                    | 3.27 × 10^{-2}|
| GO:0010605                | Negative regulation of macromolecule metabolic process    | 6                    | 3.60 × 10^{-2}|
| GO:1901700                | Response to oxygen-containing compound                     | 11                   | 3.64 × 10^{-2}|
| GO:0010033                | Response to organic substance                             | 13                   | 4.12 × 10^{-2}|
| GO:0019438                | Aromatic compound biosynthetic process                    | 18                   | 4.27 × 10^{-2}|
| GO:0033993                | Response to lipid                                         | 7                    | 4.52 × 10^{-2}|
| GO:0009892                | Negative regulation of metabolic process                  | 6                    | 4.75 × 10^{-2}|
| GO:0010556                | Regulation of macromolecule biosynthetic process          | 16                   | 4.86 × 10^{-2}|

Table 2. List of GO terms of genes upregulated more than 2-fold in HRE1β OXs.

| GO Term                   | Description                                               | Number in Input List | p-Value       |
|---------------------------|------------------------------------------------------------|----------------------|---------------|
| GO:0009636                | Response to toxic substance                               | 17                   | 2.52 × 10^{-11}|
| GO:1901700                | Response to oxygen-containing compound                     | 60                   | 8.75 × 10^{-9}|
| GO:0019748                | Secondary metabolic process                               | 29                   | 2.76 × 10^{-7}|
| GO:0009404                | Toxin metabolic process                                   | 10                   | 6.78 × 10^{-7}|
| GO:0009407                | Toxin catabolic process                                   | 9                    | 1.16 × 10^{-6}|
| GO:0090487                | Secondary metabolite catabolic process                     | 9                    | 1.16 × 10^{-6}|
| GO:001101                 | Response to acid chemical                                 | 43                   | 6.89 × 10^{-6}|
| GO:0051707                | Response to another organism                              | 44                   | 9.27 × 10^{-6}|
| GO:0043207                | Response to external biotic stimulus                      | 44                   | 9.59 × 10^{-6}|
| GO:0096607                | Response to biotic stimulus                               | 45                   | 1.29 × 10^{-5}|
| GO:0096605                | Response to external stimulus                             | 53                   | 1.49 × 10^{-5}|
| GO:0006749                | Glutathione metabolic process                             | 9                    | 2.63 × 10^{-5}|
| GO:0034614                | Cellular response to reactive oxygen species              | 8                    | 2.78 × 10^{-5}|
| GO:0071555                | Cell wall organization                                    | 25                   | 4.92 × 10^{-5}|
| GO:1901698                | Response to nitrogen compound                             | 17                   | 5.02 × 10^{-5}|
| GO:0006952                | Defense response                                          | 50                   | 8.59 × 10^{-5}|
| GO:0045229                | External encapsulating structure organization             | 25                   | 1.29 × 10^{-4}|
| GO:0022213                | Defense response to insect                                | 5                    | 2.30 × 10^{-4}|
| GO:0071554                | Cell wall organization or biogenesis                      | 28                   | 2.71 × 10^{-4}|
| GO:0010039                | Response to iron ion                                      | 7                    | 3.12 × 10^{-4}|
| GO:0071732                | Cellular response to nitric oxide                         | 5                    | 3.55 × 10^{-4}|
| GO:0006790                | Sulfur compound metabolic process                         | 19                   | 3.91 × 10^{-4}|
| GO:0010033                | Response to organic substance                             | 57                   | 3.93 × 10^{-4}|
| GO:0006575                | Cellular modified amino acid metabolic process            | 10                   | 3.94 × 10^{-4}|
| GO:0010035                | Response to inorganic substance                           | 32                   | 4.05 × 10^{-4}|

We further analyzed the downregulated genes in the HRE1α OXs and HRE1β OXs. Genes downregulated in HRE1α OXs, but not in HRE1β OXs, were enriched in the regulation of biological processes, such as response to salicylic acid, response to jasmonic acid, cellular response to acid chemical, proteasomal protein catabolic process, response to lipid, response to nitrogen compound, and regulation of macromolecule biosynthetic process (Table 3). However, genes downregulated in HRE1β OXs, but not in HRE1α OXs, were enriched in the regulation of biological processes such as response to abiotic stimulus, plant-type cell wall organization, phosphorelay signal transduction system, response to radiation, response to brassinosteroid, hydrogen peroxide catabolic process, and cellular response to ethylene stimulus (Table 4). The genes downregulated in both OXs were enriched in biological processes involved in response to endogenous stimulus, response to organic substance, response to hormone, response to oxygen-containing compound, and regulation of transcription (Tables 3 and 4). These results indicated that HRE1α and HRE1β might be involved in both similar and different signal transduction pathways.
were used for this analysis. Among the 85 genes upregulated in HRE1α genes, namely, conditions was analyzed using Genevestigator. Two anoxia experiments and eight hypoxia experiments

| GO Term | Description | Number in Input List | p-Value |
|---------|-------------|----------------------|---------|
| GO:0010033 | Response to organic substance | 14 | 1.57 × 10^{-4} |
| GO:0009719 | Response to endogenous stimulus | 13 | 1.61 × 10^{-4} |
| GO:1901700 | Response to oxygen-containing compound | 12 | 2.36 × 10^{-4} |
| GO:0009725 | Response to hormone | 11 | 1.59 × 10^{-3} |
| GO:001101 | Response to acid chemical | 9 | 2.49 × 10^{-3} |
| GO:014070 | Response to organic cyclic compound | 5 | 7.19 × 10^{-3} |
| GO:0009751 | Response to salicylic acid | 4 | 9.32 × 10^{-3} |
| GO:0009753 | Response to jasmonic acid | 4 | 9.81 × 10^{-3} |
| GO:006655 | Regulation of transcription, DNA-template | 12 | 1.29 × 10^{-2} |
| GO:2001141 | Regulation of RNA biosynthetic process | 12 | 1.30 × 10^{-2} |
| GO:1903506 | Regulation of nucleic acid-templated transcription | 12 | 1.30 × 10^{-2} |
| GO:051252 | Regulation of RNA metabolic process | 12 | 1.46 × 10^{-2} |
| GO:071129 | Cellular response to acid chemical | 5 | 1.61 × 10^{-2} |
| GO:019219 | Regulation of nucleobase-containing compound metabolic process | 12 | 1.70 × 10^{-2} |
| GO:043161 | Proteasome-mediated ubiquitin-dependent protein catabolic process | 5 | 1.90 × 10^{-2} |
| GO:097659 | Nucleic acid-templated transcription | 12 | 1.92 × 10^{-2} |
| GO:010149 | Proteasome protein catabolic process | 5 | 4.93 × 10^{-2} |
| GO:032774 | RNA biosynthetic process | 12 | 1.95 × 10^{-2} |
| GO:071310 | Cellular response to organic substance | 7 | 1.96 × 10^{-2} |
| GO:033995 | Response to lipid | 6 | 2.05 × 10^{-2} |
| GO:190266 | Response to nitrogen compound | 4 | 2.22 × 10^{-2} |
| GO:2000112 | Regulation of cellular macromolecule biosynthetic process | 12 | 2.57 × 10^{-2} |
| GO:010556 | Regulation of macromolecule biosynthetic process | 12 | 2.63 × 10^{-2} |
| GO:051171 | Regulation of nitrogen compound metabolic process | 12 | 3.19 × 10^{-2} |
| GO:032670 | Cellular response to hormone stimulus | 6 | 3.36 × 10^{-2} |

Table 3. List of GO terms of genes downregulated more than 2-fold in HRE1α OXs.

| GO Term | Description | Number in Input List | p-Value |
|---------|-------------|----------------------|---------|
| GO:0009628 | Response to abiotic stimulus | 37 | 3.49 × 10^{-3} |
| GO:0009719 | Response to endogenous stimulus | 33 | 8.07 × 10^{-4} |
| GO:0101033 | Response to organic substance | 35 | 2.57 × 10^{-4} |
| GO:0009725 | Response to hormone | 30 | 3.11 × 10^{-4} |
| GO:0009061 | Anaerobic respiration | 4 | 3.65 × 10^{-4} |
| GO:1901700 | Response to oxygen-containing compound | 27 | 1.42 × 10^{-3} |
| GO:0009664 | Plant-type cell wall organization | 7 | 2.97 × 10^{-3} |
| GO:0001101 | Response to acid chemical | 21 | 4.14 × 10^{-3} |
| GO:0006979 | Response to oxidative stress | 12 | 4.59 × 10^{-3} |
| GO:032780 | Cellular response to hormone stimulus | 17 | 5.09 × 10^{-3} |
| GO:0009146 | Response to light stimulus | 15 | 5.70 × 10^{-3} |
| GO:0071495 | Cellular response to endogenous stimulus | 17 | 6.31 × 10^{-3} |
| GO:0000160 | Phosphorelay signal transduction system | 8 | 6.31 × 10^{-3} |
| GO:0009314 | Response to radiation | 15 | 8.13 × 10^{-3} |
| GO:0009741 | Response to brassinosteroid | 5 | 8.15 × 10^{-3} |
| GO:004274 | Hydrogen peroxide catabolic process | 5 | 8.48 × 10^{-3} |
| GO:0071669 | Plant-type cell wall organization or biosynthesis | 8 | 9.75 × 10^{-3} |
| GO:0071369 | Cellular response to ethylene stimulus | 7 | 1.16 × 10^{-2} |
| GO:0071555 | Cell wall organization | 12 | 1.21 × 10^{-2} |
| GO:010149 | Xyloglucan metabolic process | 4 | 1.33 × 10^{-2} |
| GO:002593 | Reactive oxygen species metabolic process | 6 | 1.34 × 10^{-2} |
| GO:004274 | Hydrogen peroxide metabolic process | 5 | 1.35 × 10^{-2} |
| GO:0031328 | Positive regulation of cellular biosynthetic process | 10 | 1.38 × 10^{-2} |
| GO:0009755 | Hormone-mediated signaling pathway | 10 | 1.42 × 10^{-2} |
| GO:0009891 | Positive regulation of biosynthetic process | 10 | 1.58 × 10^{-2} |

Table 4. List of GO terms of genes downregulated more than 2-fold in HRE1β OXs.

2.10. Analysis of Genes Downstream of HRE1α and HRE1β in the Hypoxia Response

To characterize the regulation of responses to hypoxia by HRE1α and HRE1β, the expression of the 85 upregulated genes in HRE1α OXs and the 470 upregulated genes in HRE1β OXs under hypoxic conditions was analyzed using Genevestigator. Two anoxia experiments and eight hypoxia experiments were used for this analysis. Among the 85 genes upregulated in HRE1α OXs, the expression of four genes, namely, APRR9, WRKY46, CNGC12, and RAB18, increased under conditions of anoxia and
hypoxia (Figure S4). Among the 470 upregulated genes in \textit{HRE1}\(\beta\) OXs, the expression of 65 genes, including \textit{HSPI7.6}, \textit{CYP81D11}, \textit{UGT73B}, \textit{SAP12}, \textit{ZAT12}, \textit{GSTU1}, and \textit{ANAC032}, increased under conditions of anoxia and hypoxia (Figure S5). The four genes in \textit{HRE1}\(\alpha\) OXs and 65 genes in \textit{HRE1}\(\beta\) OXs corresponded to 66 individual genes, which included seven glutathione S-transferase (GST) genes, four UDP-glucosyltransferase (UGT) genes, four cytochrome P450 (CYP) genes, and four WRKY genes (Figures S4 and S5). These results suggested that these genes could be involved in responses to hypoxia mediated by \textit{HRE1}\(\alpha\) and/or \textit{HRE1}\(\beta\), and that \textit{HRE1}\(\beta\) could transactivate more hypoxia-responsive genes than \textit{HRE1}\(\alpha\).

3. Discussion

Alternative splicing of pre-mRNAs allows organisms to increase their coding potential and is one of various stress response mechanisms in plants [18]. \textit{HRE1} is an \textit{Arabidopsis} AP2/ERF transcription factor, and its gene has two alternative splicing variants, \textit{HRE1}\(\alpha\) and \textit{HRE1}\(\beta\) [33]; both are involved in the response to hypoxia in \textit{Arabidopsis} [33,34]. However, their functions have not yet been compared and studied. In this study, we describe the differences between the functions of \textit{HRE1}\(\alpha\) and \textit{HRE1}\(\beta\) in the hypoxia response and root development of \textit{Arabidopsis}.

Our results showed that both \textit{HRE1}\(\alpha\) and \textit{HRE1}\(\beta\) are involved in hypoxia response and root development. RT-PCR analysis results showed that the expression of both \textit{HRE1}\(\alpha\) and \textit{HRE1}\(\beta\) increased under hypoxia conditions (Figure 2a and Figure S1). \textit{HRE1}\(\beta\) was expressed at significantly higher levels than \textit{HRE1}\(\alpha\) under hypoxia (Figure 2a and Figure S1), indicating that \textit{HRE1}\(\beta\) might have a more important role than \textit{HRE1}\(\alpha\) in the hypoxia response. In addition, both \textit{HRE1}\(\alpha\) and \textit{HRE1}\(\beta\) showed higher expression levels in the roots than in the shoots (Figure 2d), and the primary root length in both OXs was greater than that of the WT seedlings (Figure 2e,f). \textit{HRE1}\(\alpha\) showed higher expression levels in the roots than \textit{HRE1}\(\beta\) (Figure 2d), implying that \textit{HRE1}\(\alpha\) plays more important roles than \textit{HRE1}\(\beta\) in root development although both \textit{HRE1}\(\alpha\) and \textit{HRE1}\(\beta\) are involved in root development.

BLASTP and multiple alignment analyses of \textit{HRE1} orthologs showed highly conserved sequences in the N-terminal region of \textit{HRE1}\(\beta\), but not of \textit{HRE1}\(\alpha\) (Figure 1c), demonstrating that \textit{HRE1}\(\alpha\) might have evolved after \textit{Arabidopsis} diverged from related plant species. In addition, comparison of protein sequences showed that \textit{HRE1}\(\alpha\) and \textit{HRE1}\(\beta\) share the same amino acid sequences in their AP2/ERF domain and C-terminal regions, whereas they have distinct N-terminal sequences (Figure 1). While \textit{HRE1}\(\beta\) has N-degron pathway-targeted sequences, \textit{HRE1}\(\alpha\) does not (Figure 1) [35]. This result suggests that \textit{HRE1}\(\alpha\) might not be a target of the N-degron pathway, therefore, \textit{HRE1}\(\alpha\) might be more stable than \textit{HRE1}\(\beta\) as it is protected from N-degron targeted protein degradation under normal conditions.

The well conserved transactivation motifs have been identified in transcription factors in plants. AHA motif present in HD-Zip I family proteins, such as AtHB1, AtHB7, AtHB12, and AtHB13, has transactivation activity [40]. EELR motif functions as a transactivation motif in a CCCH zinc finger protein, OsLIC [41]. In AP2/ERF transcription factor family proteins, transactivation motifs are not well identified, yet, except LWSY motif and EDLL motif [8,42]. In this study, we identified three novel transactivation motifs in AP2/ERF transcription factors, \textit{HRE1}\(\alpha\) and \textit{HRE1}\(\beta\). \textit{HRE1}\(\alpha\) was previously identified to be a transcriptional activator, and its C-terminal region plays an important role in its transactivation activity [33]; however, the motif in the C-terminal region responsible for this was not previously identified. In this study, we confirmed that both \textit{HRE1}\(\beta\) and \textit{HRE1}\(\alpha\) show transactivation activity (Figures 3 and 7 and Figure S3a), having the same amino acid sequences in their AP2/ERF domain and C-terminal region (Figure 1b,c). Further analysis of the C-terminal region showed that two motifs, the EELL and LWSY-like motifs, are responsible for the transactivation activity of \textit{HRE1}\(\alpha\)/\textit{HRE1}\(\beta\) (Figures 5 and 6 and Figure S3c,d). Specifically, all four E residues in the EELL motif play major roles (Figure 6b,c and Figure S3d). The LWSY-like motif consists of MGLWS amino acids, similar to the LWSY motif (Figure 5a). The LWSY motif has been identified in several ERF and DREB/CBF proteins, such as RAP2.12 and RAP2.2, which are members of group VII of the ERF subfamily. Both RAP2.12 and RAP2.2 contain an LWSY motif at the end of their C-terminal region, and
the LWSY motif in RAP2.12 and RAP2.2 plays an important role in their transactivation activity [8]. The N-terminal region of HRE1β showed transactivation activity due to its VDDG motif, whereas that of HRE1α did not (Figure 3, Figure 4, and Figure 7 and Figure S3b). As HRE1β has one more motif involved in transactivation activity than HRE1α, we expected that HRE1β might show stronger transactivation activity than HRE1α. Indeed, the transactivation activity of HRE1β was stronger than that of HRE1α in Arabidopsis protoplasts (Figure 7), indicating that HRE1β might be more strongly involved in transcriptional regulation than HRE1α. The VDDG, EELL, and LWSY-like motifs are highly conserved among HRE1β and its orthologs (Figures 4a and 5a). To our knowledge, this study is the first to propose that the VDDG, EELL, and LWSY-like motifs represent transactivation motifs.

RNA-Seq analysis using HRE1α OXs and HRE1β OXs revealed that HRE1β transactivates more downstream genes than HRE1α (Figure 9). We speculate that this differential transactivation could be caused by the stronger transactivation activity of HRE1β and the difference in overexpression levels of HRE1α and HRE1β in their OXs (Figures 7 and 10). The results of this analysis showed that both splicing variants are involved in the regulation of abiotic stress responses (Tables 1 and 2). In addition, we found that HRE1α regulates developmental processes and metabolism of nitrogenous and aromatic compounds, whereas HRE1β is involved in biotic stress responses, cell cycle regulation, carbohydrate metabolism, and photosynthesis (Tables 1 and 2). These results suggested that both HRE1α and HRE1β might function in abiotic stress responses, while they also transactivate different downstream pathways. Although they both have the same AP2/ERF DNA-binding domain and bind to the same cis-acting element, the GCC box (Figure 8), we could not confirm whether the direct or the downstream target genes of HRE1α and HRE1β are the same. Recently, it was reported that RAP2.12 and RAP2.2, members of group VII of the ERF subfamily, bind to HRPE and directly transactivate LBD41 and PCO1 containing HRPE in their promoters whereas HRE1 (HRE1β) does not bind to HRPE [39], suggesting that downstream pathway of HRE1 would be different to that of RAP2.12 and RAP2.2.

Genevestigator analysis using genes upregulated in HRE1α OXs and HRE1β OXs showed that four genes in HRE1α OXs and 65 genes in HRE1β OXs, corresponding to 66 individual genes, were upregulated under hypoxia conditions (Figures S4 and S5). This suggested that these 66 genes might be involved in the hypoxia response of HRE1α and/or HRE1β, and also that HRE1β transactivates more hypoxia-responsive genes than HRE1α. This result, together with the higher expression of HRE1β under hypoxic stress, suggests that HRE1β plays a more important role than HRE1α in the hypoxia response of Arabidopsis.

Taken together, our study revealed that HRE1α and HRE1β, the two alternative splicing variants of HRE1, differentially transactivate downstream genes in the hypoxia response and root development of Arabidopsis.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

All Arabidopsis thaliana plants used in this study were of the Columbia ecotype. For surface-sterilization, seeds were dipped for 1 min in 70% ethanol, followed by dipping in 10 min in 1/10-diluted commercial bleach (0.4% NaOCl); then, they were washed with sterile distilled water four times. The seeds were placed in the dark for 2 days at 4 °C, and the seedlings were grown on agar plates containing salts and vitamins in half-strength MS medium [43], 2.0% sucrose, and 0.7% agar under short-day conditions (8/16 h light/dark cycles) at 22 °C. Ten-day-old seedlings were transferred to soil and grown under long-day conditions (16/8 h light/dark cycles) at 22 °C.

4.2. Plasmid Construction

To generate an HRE1β-overexpressing construct, the full-length ORF of HRE1β was amplified by PCR and then cloned into the BamHI-XbaI sites in a binary vector, pFG571 [44]. The HRE1α OXs used in this study are from the line employed in a previous study [33].
To generate constructs for the analysis of transactivation activity in yeast, full-length ORFs and partial fragments of HRE1α and HRE1β were amplified by PCR and cloned into the EcoRI-SalI sites in pBD-GAL4 in frame with GAL4 BD. To generate constructs for the analysis of the transactivation activity in Arabidopsis protoplasts, full-length ORFs and partial fragments of HRE1α and HRE1β were fused with GAL4 BD under the control of cauliflower mosaic virus (CaMV) 35S promoter.

The primers used for cloning are listed in Table S5.

4.3. Plant Transformation and Selection of Transgenic Plants

The binary vectors were introduced into Agrobacterium tumefaciens strain GV3101 (pMP90) using the freeze–thaw method [45]. Then, Agrobacterium-mediated Arabidopsis transformation was performed using the floral-dipping method [46]. Transgenic plants were selected on MS plates containing 50 mg/L kanamycin. Homozygous plants of the T3 or T4 generation were used in this study.

4.4. Stress Treatments

For hypoxia treatment, prior to RT-PCR analysis, 10-day-old WT seedlings on MS plates were transferred to filter paper saturated with MS medium and treated with 99.99% nitrogen gas under dark conditions for 0, 1, 2, 4, and 8 h. To analyze phenotypic responses to the hypoxia treatment, 30 seeds of WT plants, HRE1α OXs, and HRE1β OXs were germinated on the same MS plates. After 7 days, the plates were placed under 99.99% nitrogen gas in the dark for 24 h and then allowed to recover for 2 weeks.

4.5. Root Length Measurement

The WT plants, HRE1α OXs, and HRE1β OXs were germinated on the same MS plates. After 7 days, the plants were pulled out from the MS plates and their primary root lengths were measured.

4.6. Yeast Transformation

To investigate transactivation activities in yeast, GAL4 BD-fusion constructs were transformed into a yeast strain, YD116, which harbored GAL1::URA3 and UAS_{GAL4}::lacZ as reporter genes. The yeast transformation was carried out using Frozen-EZ Yeast Transformation II™ kit (Zymo Research Corp., Irvine, CA, USA), according to the manufacturer’s instructions. Transformants were selected on synthetic minimal media lacking tryptophan (SM-Trp).

4.7. Transactivation Activity Analysis in Yeast

For yeast growth assay, transformants selected on SM-Trp were streaked onto SM lacking tryptophan and uracil (SM-Trp/-Ura) and incubated at 30 °C for 3–5 days.

Quantitative β-galactosidase assay using ONPG as a substrate was performed according to the methods of Miller et al. [47]. The unit of β-galactosidase activity was then calculated using the formula

\[ 1000 \times \frac{OD_{420}}{OD_{600} \times \text{assay time in min} \times \text{assay volume in mL}}. \]

For β-galactosidase filter assay, the transformants on SM-Trp were analyzed using 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside as a substrate. The β-galactosidase filter assay was performed according to the Clontech Yeast Protocols Handbook (Clontech Laboratories, Inc., Mountain View, CA, USA). Reactions were carried out for 6 h.

4.8. Protoplast Transformation

Arabidopsis protoplast isolation and polyethylene glycol-mediated transformation was performed according to the method described by Yoo et al. [48]. The effector plasmid and reporter plasmid were transformed into Arabidopsis protoplasts. A reference plasmid was also cotransformed with these for the normalization of the transformation efficiency. In the transactivation activity analysis, GAL4 BD-fusion constructs under the control of CaMV 35S promoter and firefly luciferase gene under the control of
4× UAS\textsubscript{GAL4} were used for the effector plasmid and reporter plasmid, respectively. Full-length ORFs of HRE1\textalpha{} and HRE1\textbeta{} under the control of CaMV 35S promoter and firefly luciferase ORF under the control of 4× GCC box were used for the effector plasmid and reporter plasmid, respectively. Nano luciferase gene under the control of CaMV 35S promoter was used for the reference plasmid.

4.9. Dual-Luciferase Assay

Luciferase activity was quantified using the Nano-Glo® Dual-Luciferase® Reporter Assay System (Promega Corp., Madison, WI, USA) and GloMax®-Multi Detection System (Promega Corp., Madison, WI, USA), according to the manufacturer’s instructions.

4.10. RNA Isolation, cDNA Synthesis, Semi-Quantitative RT-PCR, and Quantitative RT-PCR

Total RNA was isolated using an RNAqueous Kit (Invitrogen, Carlsbad, CA, USA) with Plant RNA Isolation Aid (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. Next, 2 μg of total RNA was reverse-transcribed in a total reaction volume of 25 μL; the reaction mixture contained 0.5 μg of oligo-dT primer, 0.5 mM dNTP, 5 μL of 5× reaction buffer, and 200 U of Moloney murine leukemia virus reverse transcriptase (Promega Corp., Madison, WI, USA). Quantitative RT-PCR was performed in reaction volume of 20 μL; the reaction mixture contained 0.4 μL of cDNA, 10 μL of 2× Power SYBR Green PCR Master mix (Applied Biosystems, Foster, CA, USA), and 0.25 μM gene-specific primers using QuantStudio™ 3 real-time PCR system (Applied Biosystems, Foster, CA, USA). QuantStudio™ Design and Analysis software v.1.4.3 (Applied Biosystems, Foster, CA, USA) was used for the analysis of real-time DNA amplification. The amounts of the target genes expressed were normalized to the expression levels of GAPc. The PCR was performed as previously described [49] and all primers used are presented in Table S1.

Semi-quantitative RT-PCR was performed in a reaction volume of 50 μL; the reaction mixture contained 1 μL of cDNA, 0.5 μM gene-specific primers, 0.5 mM of dNTP, 1 U of F-taq DNA polymerase (Solgent, Daejeon, Korea), and 5 μL of 10× reaction buffer. PCR was carried out in 30 cycles for HRE1\textalpha{} and HRE1\textbeta{} and in 23 cycles for GAPc. The number of PCR cycles chosen was in the linear range of the amplification reaction. GAPc was amplified as an internal control for the normalization of target gene expression levels. The reaction consisted of an initial denaturation step at 94 °C for 5 min, followed by repeated cycles at 94 °C for 45 s, 56 °C for 45 s, and 72 °C for 45 s, and a final step at 72 °C for 10 min. The primers used in the PCR are provided in Table S1.

4.11. Multiple Alignment Analysis

The conserved amino acid sequences in HRE1\textbeta{} and its orthologs were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo) and then manually corrected.

4.12. Phylogenetic Tree

A phylogenetic tree was generated using Maximum Likelihood method in MEGA 7.0.26 software. The number on each node indicates the bootstrap value for 1000 replicates.

4.13. Library Preparation and Sequencing

RNA-Seq was carried out on samples from 10-day-old WT, HRE1\textalpha{} OX, and HRE1\textbeta{} OX whole seedlings. For control and test RNAs, libraries were constructed using QuantSeq 3′ mRNA-Seq Library Prep Kit (Lexogen, Inc., Vienna, Austria), according to the manufacturer’s instructions. In brief, 500 ng of each total RNA was prepared. Then, an oligo-dT primer containing an Illumina-compatible sequence at its 5′ end was hybridized to the RNA, and reverse transcription was performed. After degradation of the RNA template, second-strand synthesis was initiated by a random primer containing an illumina-compatible linker sequence at its 5′ end. The double-stranded library was purified using magnetic beads to remove all reaction components. The library was amplified to add the complete
adapter sequences required for cluster generation. The finished library was then purified to remove all lingering PCR components. High-throughput sequencing (single-end 75 bp sequencing) was performed with NextSeq 500 (Illumina, Inc., San Diego, CA, USA).

4.14. Data Analysis

QuantSeq 3′ mRNA-Seq reads were aligned using Bowtie2 [50]. Bowtie2 indices were either generated from the genome assembly sequences or the representative transcript sequences for the alignment of the genome and transcriptome. The alignment file was used for assembling transcripts, estimating their abundances, and detecting the differential expression of genes. DEGs were determined based on counts from unique and multiple alignments using coverage in Bedtools [51]. The read count data were processed based on the quantile normalization method using EdgeR within R [52] and using Bioconductor [53]. Statistical analyses were performed using two samples t-test (equal distribution). The complete mRNA-Seq data from this publication were submitted to the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE152166. GO annotation enrichment was performed using DAVID (http://david.abcc.ncifcrf.gov/) with default parameters. Expression analysis in Genevestigator [54] was performed based on the Affymetrix Arabidopsis ATH1 Genome Array platform using the condition search tool “perturbations”.

Supplementary Materials: Supplementary Materials can be found at http://www.mdpi.com/1422-0067/21/19/6984/s1.

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Abbreviations

| Acronym  | Description                                      |
|----------|--------------------------------------------------|
| AP2/ERF  | APETALAZ/ethylene-responsive factor              |
| BD       | DNA-binding domain                               |
| CaMV     | Cauliflower mosaic virus                         |
| DAG      | Days after germination                           |
| DEG      | Differentially expressed gene                    |
| DREB/CBF | Dehydration responsive element-binding factor/C-repeat-binding factor |
| GAPc     | Glyceraldehyde 3-phosphate dehydrogenase         |
| GO       | Gene ontology                                    |
| MS       | Murashige and Skoog                              |
| ONPG     | 2-nitrophenyl-β-D-galacto-pyranoside             |
| RAV      | Related to ABI3/VP1                              |

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