The Responses of Arabidopsis Early Light-Induced Protein2 to Ultraviolet B, High Light, and Cold Stress Are Regulated by a Transcriptional Regulatory Unit Composed of Two Elements1[OPEN]

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The Arabidopsis (Arabidopsis thaliana) Early Light-Induced Protein (ELIP) is thought to act as a photoprotectant, reducing the damaging effects of high light (HL). Expression of ELIP2 is activated by multiple environmental stresses related to photoinhibition. We have identified putative regulatory elements in an ELIP2 promoter using an octamer-based frequency comparison method, and revealed a key transcriptional regulatory unit for ultraviolet B (UV-B) radiation, HL, and cold stress responses. The unit is composed of two elements, designated as Elements A (TACACACC) and B (GGCCACGCCA), and shows functionality only when paired. Our genome-wide correlation analysis between possession of these elements in the promoter region and expression profiles in response to UV-B, HL, and cold suggests that Element B receives and integrates these multiple stress signals. In vitro protein-DNA binding assays revealed that LONG HYPOCOTYL5 (HY5), a basic domain-Leucine zipper transcription factor, directly binds to Element B. In addition, mutant analysis of HY5 showed partial involvement in the UV-B and HL responses but not in the cold stress response. These results suggest that signals for UV-B, HL, and cold stress join at Element B, which recognizes the signals of multiple transcription factors, including HY5.

Plants require light for their growth, but high light (HL; or excess light) creates an overflow of electrons from the photosynthetic electron transport system. This

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Results in the generation of reactive oxygen species (ROS), which can cause damage to cells and inhibit growth (Li et al., 2009). Land plants have evolved mechanisms to protect against HL, including reduction of antenna size, decrease of the PSII-to-PSI ratio, enhanced energy dissipation from excited chlorophylls, and accumulation of photoprotective pigments. In addition, general cellular protection mechanisms such as induction of heat shock proteins and the ROS scavenging system are also involved in protection against the effects of HL (Li et al., 2009). Some of these stress responses are regulated at the transcriptional level. For example, it has been demonstrated that HL leads to transcriptional suppression of genes encoding antenna proteins and transcriptional activation of genes encoding enzymes responsible for the biosynthesis of flavonoids (Rossel et al., 2002; Kimura et al., 2003; Yamamoto et al., 2004). Transcriptional activation of Early Light-Inducible Proteins (ELIPs), fibrillins, and filament-forming temperaturesensitive (FtsH) proteases that assist in turnover of damaged PSII is also observed. These transcriptional responses to HL are thought to reduce overdriving of the photosynthetic electron transport system and thus are important for survival and growth of plants under stress.
Cold and freezing stress can also threaten plant growth and survival. Low temperatures result in photoinhibition of PSI and PSII, disruption of photosynthetic electron transport, suppression of ATP synthesis, and inhibition of the activities of enzymes in the Calvin Cycle (Falk et al., 1996). Transcriptional responses work to relieve photoinhibition, correct imbalances between synthesis and consumption of NADPH and ATP, and maintain growth under low temperatures. In fact, cold treatment and the onset of winter have been reported to activate genes encoding ELIPs, indicating the involvement of photoprotective response by cold stress (Adamska and Kloppstech, 1994; Shimosaka et al., 1999; Zarter et al., 2006). This response is physiologically distinct from the antifreezing response to cold stress for cell protection, which is primarily regulated by the DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN (DREB)/C-REPEAT-BINDING FACTOR (CBF) pathway (Chinnusamy et al., 2007; Shinozaki and Yamaguchi-Shinozaki, 2007; Nakashima et al., 2014). Thus, there are some similarities in the response of plants to cold and HL stress (Allahverdiyeva and Aro, 2012).

Exposure to ultraviolet (UV-B) radiation also threatens plant growth and survival. The primary effect of UV-B is genotoxicity. UV-B directly damages DNA by formation of thymine dimers and indirectly through generation of ROS, which arrest transcription and replication (Tuteja et al., 2009). Thus, research has focused on activation of DNA repair mechanisms and biosynthesis of UV-protective pigments. A second effect of UV-B is the promotion of photomorphogenesis, leading to inhibition of hypocotyl elongation, opening of cotyledons, and development of chloroplasts (Li et al., 2013). A third effect of UV-B is damage to PSII, which causes photoinhibition (Teramura and Ziska, 1996). Accumulation of UV-protective flavonoids and sinapate esters are considered to be a common feature of the three types of physiological responses, while reduction of antenna proteins and activation of ELIPs (Adamska et al., 1992) are part of the photosynthetic protection response related to photoinhibition.

ELIPs were first identified during the greening of etiolated pea (Pisum sativum) seedlings (Meyer and Kloppstech, 1984). The ELIP proteins belong to the chlorophyll \(a/b\)-binding protein (CAB) superfamily, which binds both chlorophylls and carotenoids (Adamska et al., 1999). While the CAB subfamily of proteins within the larger CAB superfamily has been implicated in light harvesting, ELIP subfamily proteins appear to play a role in response to environmental stress. The idea of ELIP as an antistress protein arose from expression profiling of the ELIP genes. Some ELIP genes are activated by HL (Heddad and Adamska, 2000; Kimura et al., 2001) and UV-B (Brown et al., 2005) in Arabidopsis (Arabidopsis thaliana), by cold in pea (Adamska and Kloppstech, 1994) and wheat (Triticum aestivum; Shimosaka et al., 1999), and by drought in the resurrection plant Craterostigma plantagineum (Bartels et al., 1992). While expression profiles show some variation among species, they are consistent with the possible role of ELIPs as photoprotectants for PSII in the thylakoid membrane (Adamska and Kloppstech, 1991; Heddad et al., 2012).

The molecular mechanisms responsible for the activation of ELIP genes by environmental stresses are beginning to emerge. Genomewide studies of the response of Arabidopsis to UV-B stress have shown that ELIP1 (AT3G22840) and ELIP2 (AT4G14690) are activated by UV-B through a UV-B receptor, UVR8 (Brown et al., 2005). Both genes are also activated by HL in part through a blue light receptor, CRYPTOCHROME1 (CRY1; Kleine et al., 2007). Promoter analyses have demonstrated that regulatory elements found in ELIP1 and ELIP2 (designated as ELIP1 double SORLIP1 [novel sequences overrepresented in light-induced promoters] and ELIP2 double SORLIP1) are indispensable for activation by HL (Rus Alvarez-Canterbury et al., 2014).

The response of ELIPs to a wide range of environmental stresses raises an interesting question. Is the observed up-regulation of ELIP2 by different stresses achieved by a common signal transduction pathway starting from a common sensing mechanism, or do multiple stress signals with distinct mechanisms for stress sensing merge at the ELIP2 promoter? Understanding molecular mechanisms for ELIP activation by environmental stresses could be important in understanding the environmental regulation of photosynthetic activity in higher plants.

We recently developed a unique methodology for identifying promoter elements, utilizing frequency comparison of short nucleotide sequences present in a library of promoter regions (Yamamoto et al., 2011). Our method is simple but offers considerable advantages in terms of the accuracy and sensitivity of predictions compared with established methods such as Gibbs Sampling and Multiple Expectation Maximization for Motif Elicitation that are based upon extraction of consensus sequences (Yamamoto and Obokata, 2009). We have successfully used this method to identify previously unknown transcriptional regulatory elements for the responses of Arabidopsis to aluminum (Tokizawa et al., 2015). We have used this method to identify putative promoter sequences for ELIP2 and then tested the function of these putative regulatory elements by creating synthetic promoters attached to a luciferase reporter. Our results show that the UV-B, HL, and cold stress signals merge at a single transcriptional regulatory unit in the ELIP2 promoter to give the multistress response of ELIP2 in a green tissue-specific manner. In addition, genomewide correlation analysis between promoter elements and expression profiles suggests that one of the elements unifies the multistress responses.

RESULTS

Prediction of Regulatory Elements for ELIP2 Based on Microarray Data

A survey of microarray data from experiments with Arabidopsis revealed that ELIP2 is induced between 7.8- and 96.7-fold by UV-B (Kilian et al., 2007), HL
(Yamamoto et al., 2004), and cold (Fig. 1A; Lee et al., 2005). Although hydrogen peroxide (H₂O₂) signaling is known to be involved in all these three stress responses (Brosché et al., 2010), ELIP2 showed little or no response to H₂O₂ (Fig. 1A). This indicates that the stress responses observed here are independent of H₂O₂ signaling (Kimura et al., 2001).

As a first step in our work, we made use of these microarray data to analyze the ELIP2 promoter using our octamer-based frequency comparison method (Yamamoto et al., 2011). Promoter scans that express the frequency of individual octamers in stress-induced promoters relative to their frequency in all promoters in the genome (the relative appearance ratio [RAR], degree of overrepresentation) provide a means of visualizing regions within a specific promoter that display octamers that are enriched within the population of stress-induced promoters. Calculation of RAR requires microarray data that determine a positive promoter list, global promoter list as a control, and 1-kb promoter sequences for all the promoters. Because determined RAR table covers all the possible octamers, any octamer sequences in a promoter is able to have RAR values (Fig. 1A). Because promoter regions with high RAR contain specific octamers that are enriched within the

![Figure 1](https://via.placeholder.com/150)

**Figure 1.** Prediction of regulatory elements of the ELIP2 promoter. A, Ab initio prediction of regulatory elements based upon previously published microarray data. The vertical axis (RAR) indicates the degree of concentration of an octamer frequency in the stress-responsive promoter sequences as identified to the right of each graph (Treatment) relative to the total promoter sequences in the genome. A high value means the sequence at the promoter position is more frequent in the stress-responsive promoters than it is in the library of promoters, and thus a putative regulatory element is present. Three peaks designated as A to C at the top of the graphs were identified as putative HL-responsive elements. Asterisks to the right of individual peaks indicate statistical significance determined by Fisher’s Exact Test (P < 0.05). The fold change (FC) of ELIP2 expression from the corresponding microarray data is shown to the right of each graph. The prediction with a number sign (Cold, 3 h#) was done based on a new set of 24,956 promoter sequences. B, Position (relative to the start codon), sequence, and recognition motifs of the predicted elements. For the preparation of synthetic promoters, predicted sequences (capital letters) were attached to the adjacent two bases at the 5’ and 3’ ends as indicated by small letters. B’ was not detected by prediction but identified as a related sequence of B. The underlining of B’ indicates the matched sequence with the complementary sequence of B. The sequence motif for recognition by transcription factors (TFs) is shown to the right side of each sequence. **, Morning Element (ME; CCACAC); ***, CAMTA, also known as AtSR; TSS, transcription start site.
population of stress-induced promoters, they represent putative regulatory regions.

In the case of the ELIP2 promoter, three regions (Elements A, B, and C) were detected as putative cis-elements for UV-B, HL, or cold. Element A showed significant enrichment of an octamer at position −146 in promoters that respond to HL at 150 W m\(^{-2}\), with lesser enrichment in promoters that respond to HL at 350 W m\(^{-2}\) and to cold stress. Element B showed significant enrichment of three sequential octamers at position −580 in promoters that respond to UV-B and HL, with lesser enrichment in promoters that respond to cold. An apparent enrichment in Element C (−781) in promoters that respond to HL was not significant as determined by Fisher’s Exact Test \((P < 0.05)\), but significant enrichment was observed in promoters responding to cold stress. Among the three elements, Element A shows similarity to the Morning Element, and Element C contains a known DNA motif (CGCC box) that is a target sequence of Calcium-Binding Transcription Factor (CAMTA)/Arabidopsis Signal-Responsive (AtSR) proteins (Yamamoto et al., 2011). Element B does not have high similarity to known DNA motifs; however, a closely related sequence with a one-base mismatch (designated B’) was found in the complementary strand (Fig. 1). Different from Element B, B’ contains an ACGR core sequence that is a signature of sequences targeted by basic domain-Leu zipper (bZIP) proteins (Foster et al., 1994; Jakoby et al., 2002).

Prediction-Oriented Construction of Synthetic Promoters

To understand the individual function of Elements A, B, and C, synthetic promoters were prepared. Each element was placed upstream of the TATA box from the Cauliflower mosaic virus 35S promoter (−46), followed by a firefly luciferase reporter (Fig. 2). Each element was prepared with three repeats (Supplemental Fig. S1). The effect of different combinations of two elements and the effect of all three elements combined was also subjected to analysis by preparation of combinational constructs (Fig. 2). In several cases, the order and orientation of elements were changed. These constructs were introduced into Arabidopsis for functional analysis in stable transformants.

UV-B Response

Consistent with microarray analysis (Kilian et al., 2007) and reporter assays done by other laboratories (Safrany et al., 2008), our experiments showed that a pulse UV-B under continuous white light caused a strong increase (77.2-fold: from \(8.0 \times 10^2\) at 0 h to \(6.2 \times 10^3\) cpm seedling\(^{-1}\) at 3 h) in luciferase activity in plants carrying luciferase under the control of the native ELIP2 promoter. This peak of activity occurred 3 h after the UV-B pulse (Fig. 3A). We next examined which promoter elements were able to confer this UV-B response by comparing luciferase activity in plants expressing luciferase under the control of various synthetic promoters. Plants expressing luciferase under the control of a single regulatory element showed only small (1.5- to 1.7-fold) increases in luciferase activity in response to UV-B (Fig. 3B). Absolute luciferase activities of these lines were sufficiently small that a more sensitive luminescence counter was required for detection.

In contrast to the small responses of promoters containing single elements, a large increase in luciferase activity was observed in plants expressing luciferase under the control of a combination of Elements B and A (B+A; Fig. 3C). Luciferase activity increased by as much as 65-fold after UV-B irradiation (from \(3.8 \times 10^3\) to \(2.5 \times 10^5\) cpm seedling\(^{-1}\)), a similar increase to the 77.2-fold increase observed with the native promoter. Plants expressing luciferase under the control of all three elements (C+B+A) showed only a 5.7-fold increase in luciferase activity, suggesting that Element C is a repressor of the response. Other combinations of Elements (C+A, C+B, and B+C) did not give any positive response to UV-B (Fig. 3C). These results clearly indicate that the transcriptional response to UV-B requires a combination of Elements B and A; a single element of either B or A is not sufficient to give

![Figure 2. Preparation of synthetic promoters. Triplicate sequences indicated in Figure 1B (A, B, and C) were joined with a spacer and inserted in the upstream region of the TATA-Luciferase fusion (for detailed information, see Supplemental Fig. S1). As shown, the effect of different combinations of two elements and the effect of all three elements combined was analyzed by preparation of combinational constructs. The Copy Count Unit, located upstream of each construct, was utilized for estimation of the transfer DNA copy number in the transgenic plants by competitive PCR. CaMV35S, Cauliflower mosaic virus 35S; Comp(B+A), complementary sequence of B+A.](image-url)
the response. In addition, functionality of all the predicted elements, A, B, and C, has been validated.

Table I shows that changing the order of Elements B and A from the native sequence (B+A) to A+B, or reversing the sequence (complement [B+A]), had a relatively modest effect on the UV-B response. While responses controlled by single or multiple elements that included the repressor Element C varied between 0.9- and 5.7-fold, responses controlled by combinations of Elements A and B varied between 44.7 and 80.5 (Table I). This suggested that the order and direction of the elements are not critical for their function.

We next examined the dose response to UV-B. Brown and Jenkins (2008) identified two groups of UV-B-activated genes according to their response to fluence rate. The first group responds to lower fluence and includes LONG HYPOCOTYL5 (HY5), CHALCONE SYNTHASE, and ELIP1. Their activation is mediated by an UV-B photoreceptor, UVR8. The second group responds to higher fluence rates and includes WRKY30 and UDP-glucosyltransferase 74E2. Their activation is independent from UVR8. We observed responses of the native ELIP2 promoter and the synthetic B+A promoter at fluence rates as low as 2.6 μmol m⁻² (Fig. 4). This response is comparable or more sensitive to response of HY5 as determined by a different assay system from ours (Brown et al., 2009), which may suggest that response of the ELIP2 native promoter and the synthetic B+A promoter are also regulated by UVR8. This suggestion is confirmed by an almost complete loss of UV-B response of ELIP2 in the uvr8 mutant (Brown et al., 2005).

Comparison of the native ELIP2 promoter and synthetic B+A promoter revealed a difference in their dose responses. The native ELIP2 promoter appeared to saturate at approximately 10.5 μmol m⁻². Increasing the fluence to 52.6 μmol m⁻² (Fig. 4) did not result in a significant increase in luciferase activity. By contrast, the response controlled by the B+A promoter did not show saturation under the experimental conditions we employed. This difference could be due to the increase in
copy number of Elements B and A in B+A relative to the native promoter.

The function of Elements A, B, and C were then confirmed by loss-of-function analysis. Mutations were introduced into Elements A, B, B', and C (Supplemental Fig. S2), and their response to UV-B was analyzed. Mutations in Elements A or B caused a partial loss of the UV-B response, and simultaneous mutations in Elements B and B' almost abolished the response (Supplemental Figs. S3 and S4). These results revealed that Elements B and B' are functionally redundant and are responsible for almost all of the 77.2-fold induction of the ELIP2 promoter (Fig. 3). The partial loss of response (to 37.9-fold) produced by mutation of Element A suggests that this element is functionally redundant for the UV-B response, although the corresponding element has not been identified in our analysis. Disruption of Element C increased luciferase activities at both the basal and induced levels, with the overall effect of reducing the magnitude of the UV-B response (Supplemental Fig. S4). The results under conditions of induction are consistent with the results from synthetic promoters, confirming a role of Element C as a repressor. The results at basal levels, however, are inconsistent with the results from synthetic promoters, where basal levels are elevated by addition of Element C to A+B. The reason for this inconsistence is not known now.

Taken together, these results show that all three elements detected in silico prediction using microarray data are all functional, two of which (A and B) function as a pair to control the stress response, with the third (C) acting as a repressor.

### HL and Cold Responses

Plants harboring luciferase under the control of the minimum synthetic promoter responsive to UV-B (B+A) and several other combinational constructs were subjected to HL and cold stress responses. Luciferase activity in plants with the native ELIP2 promoter showed a 10.4-fold increase after a 3-h treatment with HL (Fig. 5A) and remained at a similar level after 6 h (11.6-fold). These results are consistent with microarray data (Fig. 1) and our previous in vitro reporter analysis (Kimura et al., 2001). A negative control PC::Luciferase (LUC) that contains a native plastocyanin promoter (Vorst et al., 1993) showed a negative response.

Luciferase activity in plants harboring the B+A synthetic promoter increased 7.2-fold after 3-h treatments with HL (Fig. 5A), demonstrating that the B+A promoter is responsive to HL stress. Addition of Element C (C+B+A) reduced the response to HL, similar to what was observed in response to UV-B (Fig. 3B). The lack of response to HL in plants harboring the C+A and C+B synthetic promoters was also similar to what was observed upon exposure to UV-B. These results showed that the same minimal combination as required for the UV-B response, Element B+A, confers the HL stress response as well.

Next, we examined the cold response of the synthetic promoters. Luciferase activity in plants harboring luciferase under control of the native ELIP2 promoter increased 16.9-fold in response to 24-h cold treatment (Fig. 5B), consistent with the cold response identified by microarray analysis (Fig. 1). The peak of the cold response was between 12 to 24 h after the start of the stress treatment (data not shown), compared with the more rapid UV-B and HL responses that peaked 3 to 6 h after

### Table 1. Summary of luciferase activities at the peak (3 h) of the UV-B response

The effect of a pulse of UV-B irradiation on luciferase activity (average and SD of three or more independent transgenic lines) is shown as FC at the highest values around 3 h (2–4 h) after the UV-B pulse. For the native promoter, results of three independent assays of one representative transgenic line are shown. FCs comparable to the native promoter (38.6-fold [77.2 × 0.5] to 154.4-fold [77.2 × 2]) are highlighted in bold. All the constructs are illustrated in Figure 2.

<table>
<thead>
<tr>
<th>Construct</th>
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<th>SD</th>
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<tr>
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<tr>
<td>A</td>
<td>1.9</td>
<td>0.4</td>
</tr>
<tr>
<td>B</td>
<td>2.4</td>
<td>0.7</td>
</tr>
<tr>
<td>C</td>
<td>1.2</td>
<td>0.5</td>
</tr>
<tr>
<td>B+A</td>
<td>64.9</td>
<td>15.5</td>
</tr>
<tr>
<td>C+A</td>
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<td>0.1</td>
</tr>
<tr>
<td>C+B</td>
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<td>0.1</td>
</tr>
<tr>
<td>C+B+A</td>
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<td>1.1</td>
</tr>
<tr>
<td>B+C</td>
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<td>0.2</td>
</tr>
<tr>
<td>A+B</td>
<td>44.7</td>
<td>29.3</td>
</tr>
<tr>
<td>Comp(B+A)</td>
<td>80.5</td>
<td>18.4</td>
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</table>

**Figure 4.** Response of the native promoter and the B+A synthetic promoter to varying UV-B fluences. Response of luciferase reporters driven by the native ELIP2 and the Element B+A synthetic promoter to UV-B pulses (0.58 µmol m⁻² s⁻¹) with durations from 0 to 15 min is shown as FC. FC is calculated as a ratio of luciferase activity at the peak around 3 h (2–4 h) after UV-B pulse to the activity before the pulse (0 h). Doses of shown data are 2.6, 5.3, 10.5, and 52.6 µmol m⁻².
start of the treatments (Figs. 3 and 4). The native ELIP2 gene also showed a slow transcriptional response as revealed by real-time quantitative reverse transcription (qRT)-PCR (data not shown).

Element B+A was also able to mediate the response to cold stress, with luciferase activity increasing 13.8-fold after 24 h of cold treatment, a comparable change to that mediated by the native promoter (16.9-fold). Once again, addition of Element C suppressed the response relative to that controlled by the B+A synthetic promoter. Two control constructs, PC::LUC and 35S::LUC (Kimura et al., 2001), also showed no response (Fig. 5B).

In summary, the results from our analysis of responses mediated by synthetic promoters demonstrate that the minimal combination of elements necessary for the UV-B response, Element B+A, is also responsible for the HL and cold responses.

**Response to Circadian Rhythm of Element B+A**

Circadian oscillation of an ELIP gene was first reported in pea (Kloppstech, 1985). In our work, luciferase activity under control of the native ELIP2 promoter showed clear circadian oscillation with a progressive reduction of amplitude (Fig. 6) after a shift to continuous light. The same was true in plants expressing luciferase under the control of the B+A synthetic promoter. Interestingly, the oscillation was more robust when luciferase was expressed under control of Element B+A compared with the native promoter. The reduction in amplitude after the shift to continuous light was also not as pronounced as was observed with the native promoter. Having identified circadian oscillation in the response to light, we then used our octamer-based frequency comparison method to test for putative regulatory elements. Both Element C and Element B emerged as predicted elements for circadian oscillation (Supplemental Fig. S5), consistent with the results of our functional analysis (Fig. 6).

A summary of the response to UV-B, HL, cold, and circadian oscillation controlled by the native promoter and each of the synthetic promoters (Table II) illustrates how Element B+A can account for all the observed responses of the native promoter.

**Green Tissue-Specific Expression Mediated by Element B+A**

Many genes responding to environmental cues show tissue specificity in their expression. One could imagine
that regulation of an environmental response and tissue specificity are achieved separately, with one promoter containing an element for environmental response and another for tissue specificity. If this is true, Element B+A would not be expected to show tissue specificity because this combination contains environmental-responsive elements. We tested this hypothesis by analyzing the tissue specificity of luciferase activity under control of the native ELIP2 promoter and the B+A and C+B+A synthetic promoters using an electron multiplying (EM) CCD camera.

Luciferase activity under control of the native ELIP2 promoter (Fig. 7) showed constitutive expression around the shoot apical meristem under control conditions. This region contains young chloroplasts under development, and expression of ELIP2 is thought to be involved in protection of developing chloroplasts (Kimura et al., 2003). Strong luminescence was observed after exposure to UV-B, HL, and cold predominantly in the cotyledons, with minor expression in the roots and hypocotyls, with minor expression in the roots and hypocotyls. Tissue-specific expression in green tissues is reasonable, considering the possible role of ELIP2 as a photoprotectant for PSII. Luminescence in seedlings expressing luciferase under the control of Element B+A was also localized in green tissues. Once again, addition of Element C (C+B+A) reduced the total stress responses by differential change of the stress response among tissue. Under control conditions, seedlings expressing luciferase under the control of Element C+B+A showed luciferase expression around the shoot apical meristem that was observed with B+A (Fig. 7). Thus, the minimum combination for UV-B, HL, and cold stress responses, B+A, also confers precise tissue-specific expression, restricting the responses to green tissues.

Expression Profile of Genes Containing Elements A and B

With the knowledge that the combination of Elements B and A is the stress-responsive unit, the next question was whether the stress responses could be predicted based on specific promoter sequences. As a first step, we searched the database composed of 24,956 promoters (Hieno et al., 2014) for genes containing Elements A and B in the promoter region. A total of 236 genes containing Element A and 71 genes containing Element B in the promoter region was identified (Supplemental Table S1). The ELIP2 promoter was the only one that contained both Elements A and B. The expression profiles of these genes were analyzed using microarray data. The average responses of genes containing either Element A or B to UV-B, HL, and cold (Table III) were not significantly different than the corresponding global average. The biggest change was observed in promoters containing Element B in the UV-B response (1.70-fold versus 0.99-fold for the global average), but even this difference was not statistically significant.

Next, the percentages of positively responsive genes to the environmental stresses were calculated. The global control contained 10.4% of genes that showed a response to UV-B with more than a 2-fold increase (FC > 2; Table III). The percentage was similar (11.3%) among the genes containing Element A. By contrast, the percentage was higher (23.7%) among the genes containing Element B, suggesting a significant contribution of Element B to the UV-B response. As for the HL response, 3.9% of the global control showed a response. The percentages were much higher for genes containing

<table>
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<td>B+A</td>
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<td>A+B</td>
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<td>**</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Comp(B+A)</td>
<td>ND</td>
<td>**</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Elements A (10.8%) or B (24.5%). The global percentage of genes responding to cold stress was 6.5%, and the ratio was higher for genes containing Element B (16.9%). Therefore, genes containing Element B are more likely to respond to UV-B, HL, and cold stress. Genes containing Element A, and B', are more likely to respond to HL stress. These results suggest a more significant role for Element B on these stress responses than Element A.

During the analysis described above, we noticed that genes containing Element B tended to be genes that were responsive to all three of the stresses (UV-B, HL, and cold). Thus, we tested whether the responses to different stress were correlated. Statistically positive but very weak correlation was observed between UV-B and HL (r = 0.75; Fig. 8H) and also between HL and cold (r = 0.82; Fig. 8I). On the other hand, genes containing Element A showed statistically significant but weak correlations between UV-B and HL, UV-B and cold, and HL and cold (Fig. 8, D–F). These results revealed that Element B in the promoter region is not sufficient to produce the responses because genes with no response to the stresses are included but is sufficient for unifying these three stress responses.

**Table III. Transcriptional response of promoters containing Elements A, B, and B' to UV-B, HL, and cold**

<table>
<thead>
<tr>
<th>Gene Expression</th>
<th>Global</th>
<th>Element A</th>
<th>Element B</th>
<th>Element B'</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-B</td>
<td>−0.01 ± 1.10 (0.99)</td>
<td>0.02 ± 1.16 (1.01)</td>
<td>0.77 ± 2.23 (1.70)</td>
<td>−0.01 ± 1.00 (0.99)</td>
</tr>
<tr>
<td>FC &gt; 2</td>
<td>10.4%</td>
<td>11.3%</td>
<td>23.7%*</td>
<td>10.3%</td>
</tr>
<tr>
<td>HL</td>
<td>−0.08 ± 0.66 (0.94)</td>
<td>0.16 ± 0.90 (1.11)</td>
<td>0.74 ± 1.38 (1.67)</td>
<td>0.10 ± 0.72 (1.07)</td>
</tr>
<tr>
<td>FC &gt; 2</td>
<td>3.9%</td>
<td>10.8%*</td>
<td>24.5%*</td>
<td>7.0%*</td>
</tr>
<tr>
<td>Cold</td>
<td>−0.08 ± 0.82 (0.95)</td>
<td>0.24 ± 1.08 (1.18)</td>
<td>0.32 ± 0.95 (1.25)</td>
<td>0.03 ± 0.84 (1.02)</td>
</tr>
<tr>
<td>FC &gt; 2</td>
<td>6.5%</td>
<td>11.0%</td>
<td>16.9%*</td>
<td>7.9%</td>
</tr>
</tbody>
</table>

*Statistical significance (P < 0.05) over global percentage, judged by Fisher’s Exact Test.

We have suggested that that a combination of Elements A and B mediates the responses of ELIP2 to UV-B, HL, and cold stress. The next question is which transcription factors mediate these stress responses. One possible transcription factor is HY5, because microarray analyses have shown that ELIP2 expression is reduced in hy5 mutants (compared with the wild type) under HL and UV-B stress (Ulm et al., 2004; Brown et al., 2005; Kleine et al., 2007). To date, however, there has been no report of direct regulation of ELIP2 by HY5. Element B (GCCACGGCCA) is different from the reported HY5 target sequence (G-box: CACGTGGC; Chattopadhyay et al., 1998) and has a critical mutation in the ACGT core (Fig. 1B). Nonetheless, we examined if HY5 can bind to Element B in vitro using two different approaches.

The first approach made use of a recently developed assay (in vitro genomic DNA binding assay coupled with immunoprecipitation and next-generation sequencing [gDB-seq]) to analyze genomic fragments that bind to HY5 in vitro (Kurihara et al., 2014). In this assay, fragmented Arabidopsis genomic DNA is incubated with the HY5 protein, and fragments bound to HY5 are collected by immunoprecipitation. The genomic locus is then identified by determining the sequences of precipitated fragments. A positive signal was detected at the promoter region of direct regulation of ELIP2 by HY5.
region of \textit{ELIP2} (AT4G14690) that covers the positions of both Elements B and B’ (Fig. 9A). This assay does not give pinpoint detection at 1-bp resolution because utilized DNA fragments are approximately 150 bp long, similar to the chromatin immunoprecipitation coupled with next-generation sequencing assay.

The second approach was a luminescence-based, in vitro binding assay called AlphaScreen. In this assay, the HY5 protein and synthetic oligonucleotide probes labeled with biotin are mixed together with donor beads that produce singlet oxygen \((^{1}O_{2})\) by light excitation and acceptor beads that emit light by \(^{1}O_{2}\). Binding activity is detected as an excitation light-induced luminescence signal. The HY5 protein and a 30-bp promoter fragment containing Element B showed strong signal over control (approximately 150-fold; data not shown). Addition of nonbiotinylated competitors containing Elements B and B’ reduced the binding signal (Fig. 9B), demonstrating binding of HY5 to both Elements B and B’. By contrast, addition of the competitor with mutated Element B or nonspecific competitors containing Elements A and C did not show reduction of the binding activity of HY5 to Element B. These results show specific binding of HY5 to Element B and, with less affinity, to Element B’.

Having demonstrated specific binding of HY5 to Elements B and B’, we then examined the expression of \textit{ELIP2} in \textit{hy5} mutants by real-time reverse transcription (RT)-PCR to examine whether HY5 is also involved in the cold response of \textit{ELIP2}. Disruption of HY5 in the \textit{hy5} mutant resulted in a partial loss of the HL and UV-B responses of \textit{ELIP2} (Fig. 9C), consistent with previous reports from microarray analysis (Ulm et al., 2004; Brown et al., 2005; Kleine et al., 2007). These results, together with in vitro binding of HY5 to Elements B and B’ (Fig. 9B), lead us to conclude that HY5 directly regulates the activation of \textit{ELIP2} under conditions of UV-B and HL stress through its specific binding to Elements B and B’. The observed partial involvement of HY5 in these stress responses suggests that the role of HY5 is redundant with another transcription factor(s) that regulates the response of \textit{ELIP2} to UV-B and HL. To our surprise, the cold response of \textit{ELIP2}, which also required Element B, was not affected by the mutation of HY5 (Fig. 9C). This indicates that HY5 is not involved in the cold response of \textit{ELIP2}. 

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure8}
\caption{Possession of Elements A and B and correlation between two stress responses. The horizontal and vertical axes show transcriptional responses to indicated stresses represented in log₂(FC), where -2, 0, and 2 means FC as 0.25, 1, and 4, respectively. One dot represents one gene. The correlation coefficient is shown as \(r\) for each section. All the nine correlations shown here are statistically significant with an assumption of Student’s \(t\) test distribution.}
\end{figure}
In summary, our results strongly suggest that Element B is a target of multiple transcription factors, including HY5, with distinct physiological roles. These transcription factors build integrated stress responses, making Element B a unification point for multiple stress signals.

DISCUSSION

The response of transcription to external signals is a central part of the environmental acclimation of plants, one that is regulated by complex networks. To understand these networks, studies on individual signaling pathways from perception to gene regulation is not enough. Mechanisms for the divergence and convergence of signaling pathways should be understood as well.

Synthetic Promoter Approach to Explore Transcriptional Network

In this work, we have provided a successful example of prediction-oriented functional analysis of a plant promoter. A key factor in this success is the accuracy and sensitivity of promoter prediction. Previously, we developed a unique methodology of promoter prediction using microarray data (Yamamoto et al., 2011) and used the method to identify unique promoter elements that regulate the response of a malate transporter gene, ALUMINUM-ACTIVATED MALATE TRANSPORTERI (Tokizawa et al., 2015), to soil stresses. In this report, we utilized promoter prediction as the basis for design of synthetic promoters to identify cis-regulatory elements that can conduct environmental stress responses of ELIP2. Our results demonstrate that combining promoter prediction with an in planta assay of synthetic promoters is a practical approach.

The expression profile of ELIP2 is not simple, because multiple stress responses are observed. Nonetheless, the ELIP2 promoter contained only three predicted cis-regulatory elements for response to UV-B, HL, and cold. As a result, gain-of-function analysis using synthetic promoters was straightforward. Some promoters appear to be more complex. For example, a promoter of a transcription factor responding to a wide range of environmental and biological stresses has seven predicted cis-regulatory elements (data not shown). If we were to test all the predicted elements and all the possible pairs, we would have to prepare tens of synthetic promoters. In such cases, improvement of bioinformatics analysis to predict possible functional pairs would be helpful for future studies.

Elements A and B Form the Unit for Multiple Stress Responses

In this report, we have shown that Element B+A is sufficient to give the HL response (Fig. 5A). Statistical
analyses of genomic information suggested that Element B is the major receiver of the HL signal (Table III). Element B is related to the SORLIP1 element (GCCAC) which has been reported to be indispensable for the HL response of ELIP1 and ELIP2, as revealed by loss-of-function analysis in the native promoter (Rus Alvarez-Canterbury et al., 2014). Our results are consistent with this report; however, we have also revealed that Element B is not functional in isolation but requires a partner, Element A.

We found that Element B+A is also sufficient for the UV-B and cold stress responses (Figs. 3 and 5B; Table II). Therefore, a single unit comprised of Elements A and B receives multiple stress signals (UV-B, HL, and cold) and produces an integrated stress response. Loss-of-function analysis of the native ELIP2 promoter showed that the stress response was abolished by simultaneous disruption of Elements B and B’ (Supplemental Fig. S2), supporting an idea of a critical role for Element B (and B’) in the ELIP2 promoter.

The individual roles of Elements A and B are still to be elucidated, but our statistical analyses (Fig. 8; Table III) suggest that Element B is both the receiver and the integrator of the stress signals. By contrast, we suggest that Element A is supportive in the response. One possible role of Element A is a booster that is necessary for transcription. Another possibility is that Element A has a role as a receiver of some general or common stress signal that does not have specificity to individual stresses. The latter idea also fits with the small enrichment of stress-responsive genes containing Element A in the promoter (Table III).

Loss-of-function analyses indicate that Element B’, an element with a single base pair mismatch with Element B (Fig. 1B), is involved in the UV-B response of the native ELIP2 promoter (Supplemental Fig. S2). However, the mismatch in Element B’ occurs at a critical site. As a result, B’ acquires an ACGT motif, the core sequence recognized by proteins of the bZIP family (Foster et al., 1994; Jakoby et al., 2002). Therefore, the corresponding transactors for Element B’ may be in part different from the ones for Element B. Because the statistical analysis on the possession of elements and expression profiles (Table III) suggests that Element B’ is less specific for the stress responses, Element B’ could have additional function(s), related to bZIP factors, compared with Element B.

**Element C as a Signal Repressor**

Element C contains a CGCG motif, making it a possible target for CAMTA/AtSR transcription factors. In this report, we have shown that repression of the induced activity of the C+B+A synthetic promoter compared with the B+A promoter (Fig. 3C) and derepression of the induced activity by mutation of Element C of the native promoter compared with the native promoter (Supplemental Fig. S4). This clearly indicates that Element C acts as a repressor in the activated state. Further studies are necessary to elucidate the role of Element C in the absence of stress and the speculation that Element C might be regulated by the Ca^{2+} ion.

**Synthesis of Transcriptional Responses by Combination of cis-Regulatory Elements**

Activation of stress responses by the combination of Elements A and B evokes several pioneering reports of a synthetic response by the combination of two elements. Shen and Ho (1995) reported that a combination of ABA-Responsive Element and Coupling Element1 is necessary for the response of plants to abscisic acid. Puente et al. (1996) reported that combination of a weak light-responsive element, GATA, and a dark-responsive element, GT1, resulted in a strong light response. While these reports provided well-documented examples, the lack of further studies has limited the development of a conceptual model for the regulation of transcription by a combination of regulatory elements. Our report provides another example of a functional unit composed of two elements. As discussed previously, the asymmetric roles of Elements A and B in the stress responses is newly suggested.

Consideration of the combination of two distinct elements is also useful in the prediction of gene expression according to promoter sequences. When combinations

![Figure 10](https://example.com/image10.png)

*Coupling of two elements for functionality
*Asymmetric roles: B as possible signal receiver
*Receiving multiple environmental signals (UV-B, HL, Cold, and circadian)
*Involvement of multiple transcription factors for B
are considered, the accuracy of the prediction has been considerably improved (Zou et al., 2011). This feature may suggest that many transcriptional responses are achieved by combinations of elements.

One of our surprises in our work was the tissue specificity of the response regulated by the combination of Element B+A (Fig. 7). This means that the minimum set required for induction of the UV-B, HL, and cold responses is also sufficient to confer a tissue-specific expression profile. Similar findings were also reported for the light response, where the unit of GT1 and GATA generated a light response with cotyledon-specific expression at the seedling stage (Puente et al., 1996).

The ability of a minimum set to control both the induction and tissue specificity of a response suggests one possibility that an overall environmental response might be composed of multiple responses with different tissue specificities. Identification of transactors and their characterization will improve our understanding of this phenomenon.

Signal Unification

A common symptom of UV-B, HL, and cold stresses is excess activation of PSI and PSII and the resultant photoinhibition. Therefore, integration of these stress signals and activation of ELIP2 that plays a role in photoprotection is plausible. How might such an integrated stress response be achieved?

Recent genetic studies have shown that activation of Arabidopsis ELIP2 by UV-B is fully dependent on UVR8 (Brown et al., 2005), while activation by HL is dependent on CRY1 (at least partially; Kleine et al., 2007). The UVR8-dependent UV-B response and the CRY1-dependent HL response both require HY5 as a mediator (Brown et al., 2005; Kleine et al., 2007). Our finding that HY5 directly binds to Element B (Fig. 9) is consistent with these reports and strongly suggests direct regulation of ELIP2 by HY5. We also suggest another factor mediating UV-B response in addition to HY5 (Fig. 9). This could be another bZIP protein, HY5 HOMOLOG (HYH), because redundant regulation of ELIP1 by UVR8 through HY5 and HYH is reported (Brown and Jenkins, 2008). Brown et al. (2005) has also reported reduction of UV-B response of HY5 in hy5 mutants, and recently, HY5 is shown to be able to bind one of the promoter elements that is necessary for the UV-B response of HY5 expression, the T/G-box, in vitro (Binkert et al., 2014).

The localization of UVR8 and CRY1 in the nucleus (Brown et al., 2005; Yu et al., 2010) could be interpreted as evidence that the entirety of these signaling pathways, from signal perception of UV-B and HL to activation of the ELIP2 promoter by HY5, is located within the nucleus. However, we have previously reported that the HL response of ELIP2 is inhibited by dichlorophenyl-dimethylurea (Kimura et al., 2003) and activated by norflurazon, an inhibitor of carotenoid biosynthesis (Kimura et al., 2001). This suggests a role for the chloroplast in the HL response. These apparently conflicting results make it difficult to propose a simple working model of the signaling pathways involved in the activation of ELIP2 by these environmental stresses. One possibility might be the involvement of Element A in this feedback regulation by chloroplast activity.

Microarray data indicates that overexpression of a key regulator of the cold stress response, DREB1A/CBF3, results in a reduction in the expression of ELIP2, rather than activation (Maruyama et al., 2009). So, while the DREB/CBF pathway is a central part of the cold stress response in Arabidopsis (Chinnusamy et al., 2007), it is not involved in the cold activation of ELIP2. Some alternative regulatory process must be involved. This is consistent with the fact that neither Element A nor B (Fig. 1B) have the core sequence (CCGAC) of C-REPEAT/DEHYDRATION-RESPONSIVE ELEMENT (Stockinger et al., 1997), the target element of DREB/CBF. We have shown that HY5 is not involved in the cold stress response of ELIP2 (Fig. 5B), indicating that another transcription factor(s) plays a role in the cold response, possibly through Element B, the same element targeted by HY5. Identification of the corresponding transcription factor(s) in addition to HY5 is expected to reveal the molecular mechanism of the signal integration.

Taken together, a model of signal transduction pathways for ELIP2 activation by environmental stresses emerges (Fig. 10). UV-B/UVR8 (Ulm et al., 2004; Brown and Jenkins, 2008) and HL/CRY1 (Kleine et al., 2007) signals stimulate transcription of HY5, and HY5 binds to Element B of the ELIP2 promoter to activate transcription of ELIP2. As shown by expression analysis of the hy5 mutant, the involvement of HY5 in activation of ELIP2 by UV-B and HL is only partial (Fig. 9C). Therefore, the model provides parallel arrows with the route through HY5 from UV-B and HL to Element B. The HY5 transcription factor is not involved in the cold response (Fig. 9C); cold activation of ELIP2 is achieved independently from HY5. The clustering of stress signals at Element B fits with the expression profiles of genes containing the element (Fig. 8; Table III). The model illustrates that activation of Element B is not sufficient to elicit transcription of ELIP2, but activation of Element A is also necessary. However, the role of Element A in activating ELIP2 remains unresolved. Identification of the transcription factor(s) for this element is required to develop a more complete understanding of the role of Element A and the molecular basis of the cooperative action of Elements A and B.

MATERIALS AND METHODS

Prediction of Regulatory Elements in the ELIP2 Promoter and Bioinformatic Analysis

Microarray data from experiments testing the response of Arabidopsis (Arabidopsis thaliana) to UV-B (Kilian et al., 2007), HL (Yamamoto et al., 2004), and cold (Lee et al., 2005) were used for the prediction of regulatory elements according to our octamer-based frequency comparison method (Yamamoto et al., 2011). The promoter sequences used for prediction in this study included a set of
Preparation of Promoter::LUC Fusions

The yy446 to yy449 binary vectors containing a luciferase reporter (LUC); H.A. Naznin, Y. Yoshioka, A. Hieno, M. Hyakumachi, and Y.Y. Yamamoto, unpublished data) were used in this study. Further information about them is available at our Web site (http://www.l1g.h.u.ac.jp/~hyp/hyp/plasmid.html). A native ELIP2 promoter from -1,002 to +89 relative to the transcription start site was amplified by PCR with primers (ELIP2-F: Hind: GCC-AAG-CTT-AAT-TTA-GAA-ATT-AAC-CA-CAC-AAC-3 and ELIP2-R: 5’-CCG-CCA-TCC-TGA-GTT-TTT-CTA-AAA-GCC-GA-3’). The product was digested with HindIII/BamHI and inserted into the HindIII/BamHI site of yy448 to make yy608. Modified native promoters with mutated elements were prepared by site-directed mutagenesis using PCR with appropriate primers and introduced into yy608. Synthetic promoters were prepared by insertion of annealed synthetic oligonucleotides into the SacI/BamHI site of yy447 or HindIII/BamHI site of yy449. All constructs were checked by sequencing.

Transgenic Arabidopsis containing a plastocyanin promoter fused to a luciferase reporter gene (PC::LUC) was a generous gift from Dr. Sjef Smeekens (Vorst et al., 1993). Preparation of 35S::LUC has been described previously (Kimura et al., 2001).

Plant Transformation and Establishment of Transgenic Lines

Prepared binary vectors were introduced into Agrobacterium tumefaciens GV3101 (pMP90; Koncz and Schell, 1986) and used for transformation of Arabidopsis (ecotype Columbia) by the floral dip method (Clough and Bent, 1998). T1 seedlings showing resistance to 10 μg mL^{-1} phosphinothricin in germination medium (Valvekens et al., 1988) were subjected to competitive PCR to identify single-copy insertion lines (Yamamoto et al., 2003). At least eight independent transgenic lines were selected for each construct. Lines showing recognizable morphological disorders in the T2 generation were removed from the analysis. Eight to 24 lines were first subjected to expression analysis upon exposure to UV-B, and a representative line showing a typical response that was shared by more than one-half of the analyzed lines was selected for each construct. Irregular lines that did not show the typical response were removed from further analysis. When no representative response was obtained, additional lines were prepared and subjected to the expression analysis.

Stress Treatments and in Vivo LUC Assay

Plants were sprayed with 1% (w/v) luciferin (Dojindo) and 0.01% (v/v) Triton X100 1 d before the assay (Kimura et al., 2001) and analyzed with a photomultiplier-based, automated luminescence counter (Kishima et al., 1998). Two types of luminescence counters are available: one type assays small culture dishes with a diameter of 30 mm, while the other possesses 24 (6 × 4) photomultipliers for assays of 24-well culture plates. Unless otherwise mentioned, 13 seedlings per line were planted in a small dish and subjected to assay using the former type of photon counters. The latter type was also used to assay culture dishes with a diameter of 100 mm and provides greater sensitivity for low-luminescence samples. In this case, total counts of 24 photomultipliers were summed up for a signal of a large dish containing 60 seedlings. It should be mentioned that each photomultiplier has different sensitivity even if they have the same product number, and thus comparison between results of different photomultipliers need some caution. Luminescence images were obtained with an EM-CCD camera (C9100-13, Hamamatsu Photonics) set in a dark chamber (Kimura et al., 2003).

Eight-day-old seedlings grown on germination medium supplemented with 0.8% (w/v) Bacto agar and 1% (w/v) Suc under continuous white light (6 W m^{-2} × 15 μE m^{-2} s^{-1}) at 22°C were subjected to stress treatments. HL irradiation was achieved using a 1,000-W xenon lamp as described previously (Kimura et al., 2003). UV-B and UV-A treatments were achieved using fluorescence tubes (TL20W/12RS, Phillips Electronics) or black-light tubes (FL30SBL-B, NEC), respectively, for 15 min at a light intensity of 6 W m^{-2} measured by an UV radiometer (SERODOR, Innova). After UV treatments were complete, seedlings were returned to continuous white-light conditions. The temperature was kept at 22°C throughout the treatments. Cold treatment at -4°C was provided in a growth chamber (MIR-154-P, Panasonic Healthcare) under continuous white light at a light intensity of 6 W m^{-2}. A typical line per construct was assayed in triplicate for Figures 4 to 7.

Real-Time qRT-PCR

Total RNA was extracted from shoots of stress-treated plants of the wild type (ecotype Columbia) and transfer DNA-tagged HY5 disruptants (SALK_096651c; Alonso et al., 2003) and subjected to real-time qRT-PCR analysis as described previously (Tokizawa et al., 2015). Primers used for RT-PCR are ELIP2_F (5’-TATTGACTACACCGAACATCATGAAAAGAGATACTGTAAGTACAATCAGGATAAGATG-3’) and UBQ1_R (5’-TCTGAATGACATACGAGTAAAGTG-3’), and UBQ1_R (5’-CACCTGAAAAACAAACTCCCT-3’).

In Vitro Protein-DNA Binding Assay

In vitro translated His-Tag-HY5-human influenza hemagglutinin Tag protein was purified using Ni-agarose, checked for intactness of the preparation by immunoblotting, and subjected to gDB-seq (Kurihara et al., 2014). The amplified luminescence proximity homogeneous assay (AlphaScreen, PerkinElmer Japan) was used to determine exact binding sites of HY5. In this assay, Flag-tag-HY5 was prepared using wheat (Triticum aestivum) germ extracts (Bio Sieg, Tokushima) according to the manufacturer’s instruction. After checking intact preparations by immunoblotting using an anti-Flag antibody (Wako Pure Chemical Industries), the translation products containing wheat germ extracts were used without purification for the binding assay. A negative control of translation products without HY5 mRNA did not show any binding to the utilized DNA probe (data not shown). A biotinylated double-strand oligo DNA containing Element B (Fig. 9B, shown as B) was used as a probe of the binding assay (AlphaScreen) according to the manufacturer’s instructions in a modified buffer containing 25 mM HEPES-KOH (pH 7.6), 40 mM KCl, 0.01% (v/v) Tween 20, and 0.1% (w/v) bovine serum albumin. AlphaScreen signals, which monitor interaction between donor beads tagged with anti-Flag antibody and acceptor beads labeled with streptavidin, were expressed as relative value of luminescence signals over the one with the corresponding nonbiotinylated DNA probe. Nonbiotinylated competitor probes (Fig. 9B, shown as B, Brut, A’, A, and C) were added to examine specificity of the binding.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Sequences of synthetic promoters.

Supplemental Figure S2. Sequences of loss-of-function promoters.

Supplemental Figure S3. UV-B responses of native ELIP2 promoters with disrupted regulatory elements.

Supplemental Figure S4. Expression levels of native ELIP2 promoters with disrupted regulatory elements.

Supplemental Figure S5. Prediction of circadian responsive transcriptional regulatory elements of the ELIP2 promoter.

Supplemental Table S1. Gene list containing Elements A and B in the promoter region.

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