Heat-induced inhibition of phosphorylation of the stress-protective transcription factor DREB2A promotes thermotolerance of Arabidopsis thaliana

Plants have evolved complex systems to rapidly respond to severe stress conditions, such as heat, cold, and dehydration. Dehydration-responsive element-binding protein 2A (DREB2A) is a key transcriptional activator that induces many heat- and drought-responsive genes, increases tolerance to both heat and drought stress, and suppresses plant growth in Arabidopsis thaliana. DREB2A expression is induced by stress, but stabilization of the DREB2A protein in response to stress is essential for activating the expression of downstream stress-inducible genes. Under nonstress growth conditions, an integral negative regulatory domain (NRD) destabilizes DREB2A, but the mechanism by which DREB2A is stabilized in response to stress remains unclear. Here, based on bioinformatics, mutational, MS, and biochemical analyses, we report that Ser/Thr residues in the NRD are phosphorylated under nonstress growth conditions and that their phosphorylation decreases in response to heat. Furthermore, we found that this phosphorylation is likely mediated by casein kinase 1 and is essential for the NRD-dependent, proteasomal degradation of DREB2A under nonstress conditions. These observations suggest that inhibition of NRD phosphorylation stabilizes and activates DREB2A in response to heat stress to enhance plant thermotolerance. Our study reveals the molecular basis for the coordination of stress tolerance and plant growth through stress-dependent transcriptional regulation, which may allow the plants to rapidly respond to fluctuating environmental conditions.

As sessile organisms, plants have evolved complicated and sophisticated systems to cope with ever-changing stress conditions such as heat, cold, drought, and high light by rapidly responding to changes in their growing environment. These responses occur at the developmental, physiological, and biochemical levels, and generally, stress stimuli activate stress resistance machinery and repress growth. A large part of these responses is based on stress-induced changes in gene expression, which are regulated by specific transcription factors that bind stress-specific cis-acting elements in the promoters of stress-responsive genes (1–4). Among these stress conditions, heat and drought are closely related to each other in nature (5, 6).

Dehydration-responsive element-binding protein 2A (DREB2A) is a key transcription factor that activates the expression of many stress-inducible genes in response to both heat and drought conditions via specific binding to the cis-acting dehydration-responsive element/C-repeat and thus enhances plant tolerance to these stress conditions in Arabidopsis thaliana (7–9). The expression of DREB2A itself is induced in response to these stress conditions (7, 9), but the accumulation of DREB2A transcripts is not sufficient to activate downstream genes because of the negative post-translational regulation of DREB2A (8). Therefore, to understand how DREB2A activates downstream gene expression in response to stress, it is necessary to elucidate the mechanism by which this negative regulation is inactivated in response to stress.

The post-translational negative regulation of DREB2A involves a 30-amino acid region called the negative regulatory domain (NRD), which is located between the DNA-binding domain and the activation domain (8). The deletion of the NRD converts the DREB2A protein into a constitutively active form (DREB2A CA), and plants overexpressing DREB2A CA show...
stronger expression of many stress-inducible genes, improved tolerance to both drought and heat, and growth retardation, demonstrating the importance of negative post-translational regulation via the NRD (8, 9).

One of the functions of the NRD is to destabilize DREB2A, because DREB2A CA shows increased stability (8). Although DREB2A is unstable under nonstress conditions, it is highly and moderately stabilized in response to heat and dehydration, respectively (10, 11). The stabilization of DREB2A in response to these stresses allows the accumulation of sufficient amounts of DREB2A to induce stress-responsive target genes and is therefore a key step of stress-responsive gene expression (11). Two types of DREB2A-interacting proteins that facilitate the degradation of DREB2A via the ubiquitin-proteasome system have been identified: DREB2A-interacting proteins (DRIPs), which are C3HC4-type really interesting new gene (RING) E3 ligases (10); and BTB/POZ and MATH domains (BPMs), which act as substrate adaptors for the Cullin3 (CUL3)-based multisubunit E3 ligase (12). However, the mechanism underlying the stabilization of the DREB2A protein under stress conditions is still unknown.

Phosphorylation, as a major post-translational modification, is involved in the regulation of many proteins, and environmental stress signals are also known to induce changes in the phosphorylation of plant proteins. In response to osmotic stress, multiple protein kinases, including those of the sucrose nonfermenting 1 (SNF1)-related protein kinase 2 family (SnRK2s), the mitogen-activated protein kinases (MAPKs), and the calcium-related kinases, are activated to regulate the activity of target proteins, including transcription factors and transporters (13, 14).

In contrast, the occurrence and function of heat-induced phosphorylation changes are not well-understood. The only known heat-activated kinase is MAPK. Specific heat-activated MAPKs have been detected in alfalfa and tomato plants, and specific activation of MPK6 in response to heat has been found in A. thaliana (15–17). Reductions in phosphorylation levels in response to heat have been reported for the ribosomal protein S6 (18). In addition, the phosphorylation level of the no apical meristem, Arabidopsis transcription activation factor 1/2, cup-shaped cotyledon 2 (NAC)–type transcription factor NAC019, which is a positive regulator of heat-responsive gene expression, is slightly reduced in response to heat (19), although the functions of this phosphorylation are unknown. Phosphorylation of the heat shock transcription factor A1s (HsfA1s) can affect their function; however, the in vivo phosphorylation status of the HsfA1s remains unknown (4).

Here, we report that the DREB2A NRD is phosphorylated under nonstress conditions, and this modification is likely mediated mainly by casein kinase 1 (CK1). This phosphorylation facilitates the degradation of DREB2A and could account for the negative regulation of DREB2A stability by the NRD. Interestingly, DREB2A proteins are largely dephosphorylated under heat conditions, which coincides with the stabilization of these proteins. Thus, the NRD represents a conditional degradation signal whose function can be altered by its phosphorylation level.

Results

A Ser/Thr cluster in the N-terminal half of the NRD (N-NRD) is important for the negative regulatory function of the NRD

Removal of the NRD results in the activation and stabilization of DREB2A (8). To identify important residues in the NRD, we first collected and compared the sequences of angiosperm DREB2A orthologs. We found that many DREB2A orthologs have an acidic sequence that contains one or more clusters of Ser/Thr residues, which corresponds to the N-NRD (Fig. 1A and Fig. S1). Genome-wide searches of Ser/Thr clusters revealed that these clusters are enriched in transcription factors (Fig. 1B). Furthermore, Gene Ontology (GO) analysis revealed that the GO term “transcription factor activity” was most enriched in Ser/Thr cluster-containing proteins under the GO category molecular function, implying the significance of Ser/Thr clusters in these proteins (Fig. 1C). Deletion of both the N-NRD and an acidic sequence including Ser/Thr cluster(s) in soybean GmDREB2A;2 increased reporter activity (12, 20), suggesting functional conservation.

Then we carried out point mutation analyses of the conserved residues in the N-NRD. As previously reported (8), complete deletion of the NRD impaired its negative regulatory function and increased reporter activity (CA; Fig. 2, A and B). Single and multiple mutations of all four acidic residues in the NRD impaired its function (i.e. elevated reporter activity), but these effects were smaller than that of CA (Fig. 2, A and B). Thereafter, we selected five of the eight Ser/Thr residues in the N-NRD and mutated each of them to Ala or Asp, considering the position of the phosphorylation sites (Fig. 2A). Single mutation of these residues also impaired NRD function, but the effects of these mutations were not as strong as that of CA, suggesting that none of these Ser/Thr residues are critically important for negative regulation (Fig. 2C).

Based on this result, we considered that Ser/Thr residues in the N-NRD might be potential targets of multiple phosphorylation. Hence, we introduced simultaneous mutations at four or five of these residues in different patterns (Fig. 2D). In reporter assays, all the Ala mutations greatly impaired NRD function, (Fig. 2D). Of them, the 5A-3 mutation, in which five sequential Ser/Thr residues were replaced with Ala, fully impaired NRD function at a level comparable with that caused by the complete deletion of the NRD (CA) (Fig. 2D). For this reason, we selected the 5A-3 mutant for further research. Interestingly, the replacement of the same five sequential Ser/Thr residues with Asp (5D-3) significantly reduced reporter activity compared with the effects of 5A-3, and the effect on reporter activity was the weakest of all the mutations tested (Fig. 2D). As a counterpart of the 5A-3 mutation, we additionally selected the 5D-3 mutation, and the other mutants were not studied further.

In agreement with the reporter activity, the protein level of 5A-3 was comparable with that of CA and higher than that of 5D-3 (Fig. 2E). Collectively, the results obtained in the transient expression system indicate that Ser/Thr residues in the N-NRD, especially those in the conserved core clusters of Ser/Thr, are important for the function of the NRD.
Ala and Asp mutations of the Ser/Thr cluster affect the expression of stress-inducible genes differently under the control condition

To confirm the results obtained in the transient assay system, we generated transgenic *A. thaliana* plants that constitutively expressed the full-length (FL), CA, 5A-3, and 5D-3 forms of DREB2A in the *dreb2a-1* mutant background. We selected two lines that showed similar levels of transgene expression for each construct and measured the expression levels of several stress-inducible genes downstream of DREB2A in these transgenic plants (Fig. 3A). We used genes exhibiting different expression patterns: *At4g36010* is induced in response to both heat and dehydration, whereas *HsfA3* and *Hsp70* are specifically induced in response to heat, and *RD29A* and *RD29B* are specifically induced in response to dehydration. As previously reported (20), these genes exhibited higher expression in the CA plants than in the FL plants (Fig. 3A). The expression levels of these genes were also elevated in 5A-3 plants relative to FL plants (Fig. 3A). In contrast, the expression levels of these genes in 5D-3 plants were as low as those in the FL plants, which was significant for the three heat-inducible genes, *At4g36010*, *HsfA3*, and *Hsp70* (Fig. 3A).

Next, we analyzed the levels of DREB2A protein accumulation in these transgenic plants (Fig. 3B). In the presence of the proteasome inhibitor MG132, the amounts of each form of the DREB2A protein were similar, reflecting the expression levels of each transgene. Consistent with the results of the gene expression analysis, the 5A-3 and CA proteins showed higher accumulation than the FL protein in the absence of MG132, suggesting increased stability of these proteins (Fig. 3B). In contrast, the 5D-3 protein showed small increases relative to the FL protein, suggesting that the 5D-3 protein remained unstable (Fig. 3B).

Finally, we tested whether the observed changes in the expression levels of stress-inducible genes were associated with stress tolerance. We checked the thermotolerance of these plants, because the stabilization and resultant accumulation of the DREB2A protein are more drastic during responses to heat
than during responses to dehydration (11). The results showed that the 5A-3 plants showed significantly higher levels of thermotolerance than the 5D-3 plants, which did not show improved thermotolerance (Fig. 3, C and D). These results confirmed that the core Ser/Thr cluster in the N-NRD is important for the negative regulatory function of the NRD. The contrasting effects of the 5A-3 and 5D-3 mutations suggest an involvement of phosphorylation in the regulation of NRD function, in which phosphorylation of the Ser/Thr cluster plays a role in the negative regulation of DREB2A stability by the NRD.

**DREB2A is phosphorylated in vivo, and the NRD contains major phosphorylation sites**

To test whether DREB2A is phosphorylated *in vivo*, we analyzed the phosphorylation levels of DREB2A under the control condition in the FL and CA plants. We used immunoblot analysis combined with Phos-tag PAGE, in which phosphorylated proteins are detected as upshifted bands (21). The FL protein that accumulated after treatment with the proteasome inhibitor MG132 was detected as a single major band on an SDS-PAGE gel, whereas shifted bands that represented putative phosphorylation forms were detected on a Phos-tag PAGE gel (Fig. 4A). In contrast, the CA protein was mainly detected as a nonshifted band on the Phos-tag PAGE gel regardless of the MG132 treatment, although a smaller number of shifted bands were still detected after MG132 treatment (Fig. 4A).

The shifted bands were confirmed to be phosphorylated forms of DREB2A by treating the protein sample with protein phosphatase (Fig. 4B). The densitometric scan data of the Phos-tag PAGE gel image showed that the FL protein had broad low-

![Figure 2. The Ser/Thr cluster in the NRD is important for negative regulation.](image-url)

**Figure 2. The Ser/Thr cluster in the NRD is important for negative regulation.** A, schematic diagrams of the constructs used for transient expression in *A. thaliana* mesophyll protoplasts. The effector plasmid contained either a FL, CA, or point-mutated DREB2A coding sequence, which was expressed under the control of the cauliflower mosaic virus 35S promoter (35S-pro, a constitutive promoter), the tobacco mosaic virus omega sequence (35S-omega, a translational enhancer) (61), and the Agrobacterium nopaline synthase terminator (NOS-T). The positions of the DNA-binding domain (BD) and the NRD are indicated. The reporter plasmid contained the β-glucuronidase (GUS) coding sequence, which was fused downstream of three dehydration-responsive elements (DRE X3) and the RD29A minimal promoter (TATA) (7). Internal control plasmids contained either luciferase (LUC)- or GFP-coding sequences. B–D, effects of single or multiple mutations of Asp/Glu (B), single mutations of Ser/Thr (C), and multiple mutations of Ser/Thr (D) on the transactivation of the reporter gene in protoplasts. The values indicate reporter activity, which was obtained by normalizing GUS activity with LUC activity. The mean value that was obtained with the empty effector plasmid was set to 1. The bars indicate the mean and S.D., and the points above each bar represent individual data points. Means with different letters are significantly different (Tukey–Kramer multiple comparisons test, *p* < 0.05). E, effects of the mutations on the stability of the DREB2A protein. Protoplasts expressing DREB2A proteins were treated with MG132 for 1 h, and DREB2A protein levels were analyzed by immunoblot. Four biological replicates were pooled as one protein sample. A representative result from three independent experiments is shown.
mobility peaks of phosphorylated forms that were stronger than the peak of the nonphosphorylated form, whereas the CA protein had a dominant strong peak of the nonphosphorylated form (Fig. 4C). A comparison of the band intensities before and after phosphatase treatment indicated that 80% of the FL protein was phosphorylated, whereas approximately only 20% of the CA protein was phosphorylated (Fig. 4B). These results indicate that DREB2A is phosphorylated under the control condition and suggests that the phosphorylated forms of DREB2A are less stable than the nonphosphorylated form. Furthermore, these results demonstrate that the NRD is important for phosphorylation, although minor phosphorylation outside the NRD also occurs.

Next, we located the phosphorylation sites within the NRD. In addition to the five sequential Ser/Thr residues that were mutated in 5A-3 and 5D-3, the N-NRD contains three other Ser/Thr residues. We made mutant forms of DREB2A in which these three residues and all eight Ser/Thr residues were simultaneously mutated into A and D (3A/3D and 8A/8D, respectively) (Fig. 4D) and transiently expressed these mutant DREB2As in protoplasts. Similar to the results obtained using transgenic plants, the FL protein in MG132-treated protoplasts was detected as shifted bands representing the phosphorylated forms of FL on the Phos-tag PAGE gel, whereas the CA protein was mainly detected as the nonphosphorylated form (Fig. 4, E and F). The 5A-3 protein was mainly detected as the nonphosphorylated form in both protoplasts and transgenic plants (Fig. 4, E and F, and Fig. S2), suggesting that the five sequential Ser/Thr residues are important for phosphorylation.

In contrast to the 5A-3 protein, a large part of the 5D-3 protein remained phosphorylated in the presence of MG132 (Fig. 4, E and F, and Fig. S2). This finding implies that the phosphorylation of DREB2A by phosphorylation

Figure 3. Ala and Asp mutations of the Ser/Thr cluster had different effects on the expression of stress-inducible genes under the control condition in transgenic plants. A, the mRNA levels of the transgene and DREB2A downstream genes in transgenic A. thaliana plants constitutively expressing mutant DREB2A proteins. Lowercase letters above the construct name indicate the name of the individual transgenic line. The values indicate the mean levels of transcripts normalized to ACTIN2, and the error bars indicate S.D. (n = 3). Expression levels in the CA plants were set to 100. The RNA samples were obtained from six to eight T3 plants. Means with different letters are significantly different (Tukey–Kramer multiple comparisons test, p < 0.05). VC, vector control. B, DREB2A protein levels in transgenic plants treated without or with MG132. Histone H3 protein levels and the Rubisco large subunit (RbcL) stained with Ponceau S are shown to indicate equal loading. Half and quarter amounts of the CA (line a) samples were loaded in the two right lanes to show the effectiveness of the loading controls. The protein samples were obtained from six to eight T3 plants. A representative result from two independent experiments is shown. C and D, thermotolerance of transgenic A. thaliana plants. Images of plants (C) and their chlorophyll contents (D) after 80 min of heat shock at 42 °C and 7 days of recovery at 22 °C are shown. The image shown in C is a representative result of three independent experiments. The values in D indicate chlorophyll content relative to fresh weight. The bars indicate the mean and S.D., and the points to the left of each bar indicate individual data. The data were obtained from more than three independent experiments, and one experiment contained 15 plants/line. Means with different letters are significantly different (Tukey–Kramer multiple comparisons test, p < 0.05).
ylation in the sequential five Ser/Thr residues positively influenced the phosphorylation of other sites. Similarly, a relatively large portion of the 3D protein remained phosphorylated compared with the 3A protein, suggesting that phosphorylation within these three residues also positively affects the phosphorylation of other Ser/Thr residues (Fig. 4, E and F). In contrast to these proteins, the 8A and 8D proteins showed similar phosphorylation patterns characterized by a high proportion of the nonphosphorylated form (Fig. 4, E and F). These results suggest that these eight Ser/Thr residues include those that are important for the phosphorylation of DREB2A.

In summary, our data suggest that multiple sites in the NRD are phosphorylation targets and that phosphorylation within the NRD may positively affect the phosphorylation of other phosphorylation sites. MG132 treatment increased the ratio of phosphorylated to nonphosphorylated forms for all DREB2A proteins, further supporting that the phosphorylated forms were less stable than the nonphosphorylated form (Fig. 4E).

Although the 8D protein was more stable than the 5D-3 protein in the absence of MG132, this difference in stability may have been due to the substitution of nonphosphorylated Ser/Thr residues in 8D.

**DREB2A is phosphorylated by nuclear kinases**

We previously showed that nuclear localization is necessary for the degradation of DREB2A (11). To test whether nuclear localization is also necessary for the phosphorylation of DREB2A, we compared phosphorylation patterns between GFP-DREB2A FL (GFP-FL) and GFP-DREB2A Δ1/2 (GFP-ΔNLS), which is stable and deficient in nuclear localization (Fig. 5A) (11). In transgenic plants expressing these proteins, GFP-ΔNLS accumulated at a high level, and its level did not change in the presence of MG132 (Fig. 5B). Phos-tag PAGE analysis of these samples showed that the majority of GFP-ΔNLS was not phosphorylated, whereas the majority of GFP-FL was phosphorylated in vivo. A phosphorylation of DREB2A under the control condition. Protein samples extracted from DREB2A-overexpressing plants either without or with MG132 treatment were separated using SDS-PAGE and Phos-tag PAGE gels and detected by immunoblot. For each construct, line a shown in Fig. 3 was used. DREB2A-P and DREB2A-NP in the Phos-tag PAGE gel indicated shifted bands of the putative phosphorylated forms and nonshifted bands of the putative nonphosphorylated forms of DREB2A, respectively. Asterisks indicate nonspecific bands. RbcL stained with Ponceau S is shown as a loading control. Each protein sample was obtained from eight T3 plants. VC, vector control. B, shifted bands identified as phosphorylated DREB2A. Protein samples of MG132-treated transgenic plants in A were treated with or without λPP, and the phosphorylation levels were analyzed using Phos-tag PAGE. The ratios of the DREB2A-NP signal intensities obtained without λPP relative to those obtained with λPP are indicated. Asterisks indicate nonspecific bands. The same samples were also analyzed using an SDS-PAGE gel. C, densitometric scan data of the Phos-tag PAGE gel image shown in B. For each construct, scan data obtained with and without λPP were superimposed on the same panel. D, a schematic image of the constructs that have additional replacements of Ser/Thr residues in the NRD. E, phosphorylation levels of the various mutant DREB2A proteins in protoplasts. DREB2A proteins that were transiently expressed in protoplasts in the absence or presence of MG132 were separated using SDS-PAGE and Phos-tag PAGE gels and detected by immunoblot. Two biological replicates were pooled as one protein sample. F, densitometric scan data of the Phos-tag gel image in E. MG132-treated samples were analyzed. All data represent results from more than two independent experiments.
Regulation of DREB2A by phosphorylation

Next, we tested whether nuclear kinases could phosphorylate DREB2A by using an in vitro assay system. We used GST-tagged DREB2A fragments that were expressed in Escherichia coli as substrates and a nuclear protein fraction prepared from Arabidopsis thaliana plants as a kinase source (Fig. 6A). A GST-tagged protein of DREB2A FL was not included because the expression of this protein was not successful in E. coli. After incubating these samples with \([\gamma-^{32}P]ATP\), phosphorylated proteins were detected by autoradiography. All tested fragments were phosphorylated by the nuclear protein fraction, but it is noteworthy that the NRD fragment, which contained only the NRD sequence, was phosphorylated (Fig. 6B). Although the results of in vivo phosphorylation analyses showed the importance of NRD in the phosphorylation of DREB2A (Fig. 4), the C-terminal 166–335 fragment was more strongly phosphorylated than the NRD in vitro (Fig. 6B). We speculate that the fragmentation of DREB2A might affect the affinity of responsible kinases for certain fragments such as the NRD, which could reduce phosphorylation rates.

To screen for the kinase families that are responsible for the phosphorylation of DREB2A, we also tested the effects of protein kinase inhibitors on the in vitro phosphorylation of DREB2A (Fig. 6B). The Ser/Thr cluster of DREB2A contains consensus sequences recognized by glycogen synthase kinase 3 (GSK3) [(S/T)XX(pS/pT), where pS is phosphoserine, and pT is phosphothreonine], CK1 ((D/E)XX(S/T)) or (pS/pT)XX(S/T)) and casein kinase 2 (CK2) ((S/T)XX(D/E) or (S/T)XX(pS/pT)) (22, 23). Therefore, we used bikinin (24) and heparin (25, 26), which inhibit A. thaliana GSK3 and CK1/CK2 in vitro, respectively. Stauroporin was used as a protein kinase inhibitor with broad specificity.

The in vitro phosphorylation of the NRD, 136–335, and 166–335 fragments was highly sensitive to heparin (Fig. 6B), suggesting the involvement of casein kinases in the phosphorylation of DREB2A in both the NRD and the C-terminal region. In contrast, heparin exerted slight and no effects on the phosphorylation of the 1–165 and 1–135 fragments, respectively (Fig. 6B), suggesting that heparin-insensitive kinases played major roles in the phosphorylation of these fragments in the in vitro system. Although the 1–165 fragment contains the NRD, it might not be a good substrate for casein kinases, possibly because of a reduction of affinity caused by fragmentation.

Next, using MS, we identified the phosphorylation sites in the 136–335 fragment (Fig. 7). This fragment was selected because it contains the NRD and strongly phosphorylated in vitro (Fig. 6B). As a result, six in vitro phosphorylation sites were identified, and these sites were considered to be potential in vivo phosphorylation sites (Fig. 7). Four of these sites were identified in the N-NRD, whereas two were identified in the C-terminal region (Fig. 7). The identification of these four phosphorylation sites in the N-NRD in vitro suggests the phosphorylation of these Ser/Thr residues in vivo. The two sites identified outside the NRD might be related to residual phosphorylation in DREB2A CA (Fig. 4, B and C).

CK1-mediated phosphorylation of the NRD facilitates degradation of DREB2A

Next, we tested whether the inhibition of casein kinases affected the stability and phosphorylation level of DREB2A in vivo. Because heparin is not cell-permeable and has the potential of causing secondary effects by nonspecific binding to acidic proteins, we selected cell-permeable inhibitors that are selective to either CK1 or CK2. First, we treated transgenic A. thaliana plants constitutively expressing DREB2A FL or CA with PF-670462, which is a highly CK1-selective inhibitor (27). The PF-670462 treatment caused the accumulation of DREB2A FL in a dose-dependent manner (Fig. 8A). Phos-tag PAGE analysis showed that the accumulated FL protein mainly existed as the nonphosphorylated form (Fig. 8A).

To visualize the unstable phosphorylated forms, the phosphorylation level of DREB2A was also analyzed in the presence of MG132 (Fig. 8B). The phosphorylation level of DREB2A was

Figure 5. Nuclear localization is necessary for the phosphorylation and destabilization of DREB2A. A, schematic diagrams of GFP-DREB2A proteins that were constitutively expressed in transgenic plants. The ΔNLS mutant lacks both nuclear localization signals, and the nuclear localization of this mutant is impaired (11). VC, vector control. B, stability of the GFP-FL and GFP-ΔNLS proteins. Protein samples were prepared from transgenic A. thaliana plants that expressed GFP-FL or GFP-ΔNLS proteins and were analyzed by immunoblotting using an SDS-PAGE gel and the anti-DREB2A antibody. Because the GFP-ΔNLS protein accumulated at a high level, \(1/10\) volumes of the GFP-ΔNLS samples were simultaneously analyzed on the same gel. C, phosphorylation level of GFP-FL. The samples used in B were analyzed using a Phos-tag PAGE gel as in Fig. 4B. Low- and high-contrast images of the same blot are shown.

Figure 6. Stability and phosphorylation level of DREB2A FL and CA with various cell permeable inhibitors. A, schematic diagrams of GFP-DREB2A proteins, the low- and high-contrast images of the Phos-tag PAGE for the phosphorylation level of DREB2A FL and CA with various cell permeable inhibitors. B, stability of the GFP-FL and GFP-ΔNLS proteins and were analyzed by immunoblotting using an SDS-PAGE gel and the anti-DREB2A antibody. Because the GFP-ΔNLS protein accumulated at a high level, \(1/10\) volumes of the GFP-ΔNLS samples were simultaneously analyzed on the same gel. C, phosphorylation level of GFP-FL. The samples used in B were analyzed using a Phos-tag PAGE gel as in Fig. 4B. Low- and high-contrast images of the same blot are shown.
greatly reduced by treatment with PF-670462, suggesting that PF-670462 treatment inhibited the phosphorylation of DREB2A. Although PF-670462 also reduced the minor phosphorylation of DREB2A CA (Fig. 8B), the effects of PF-670462 on the total amount of DREB2A CA were small (Fig. 8A), suggesting that the major phosphorylation of the NRD is more important for DREB2A destabilization than the minor phosphorylation of the other regions.

We also tested the effects of CK2 inhibition. In contrast to PF-670462, the CK2 inhibitor 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB) did not increase the amounts of DREB2A FL and CA (Fig. 8C), nor did it significantly reduce the phosphorylation levels of DREB2A FL and CA (Fig. 8D). Collectively, these in vitro and in vivo inhibitor experiments suggest that CK1 is probably responsible for the phosphorylation of the NRD, which in turn facilitates the degradation of DREB2A (Figs. 6 and 8).

The increase in the stability of DREB2A in response to heat is associated with a reduction in its phosphorylation level

We have previously reported that the DREB2A protein is highly stabilized in response to heat (11). We therefore tested whether the phosphorylation of DREB2A changes in response to heat using transgenic A. thaliana plants constitutively expressing DREB2A FL. We observed the phosphorylation level of the DREB2A that accumulated in response to heat stress in the absence of MG132 and found that the majority of the accumulated DREB2A was of the nonphosphorylated form (Fig. 9A and B). In the presence of MG132, the phosphorylated forms were dominant before exposure to heat stress, but molecular species with low phosphorylation levels became dominant after exposure to heat stress (Fig. 9A and B). These results demonstrate that the increased stability of DREB2A in response to heat is associated with a decreased phosphorylation level.

We also analyzed the relationship between stability and phosphorylation level at different temperatures. At 40 °C, DREB2A accumulated at a high level and was not phosphorylated, whereas at 36 °C, the accumulation of DREB2A was moderate, and phosphorylated forms were produced after 2 h of heat stress (Fig. 9C). No change in phosphorylation level was observed at 32 °C, and stabilization of DREB2A did not occur (Fig. 9C). Therefore, a parallel relationship exists between the phosphorylation level and stability of DREB2A at different levels of heat stress.

Finally, we asked whether the reduction of the phosphorylation level was due to a reduced phosphorylation rate of newly synthesized DREB2A or an increase in phosphatase activity toward DREB2A. We hypothesized that if an increase in phosphatase activity were the main cause of the reduced phosphorylation level, then the phosphorylated DREB2A proteins that accumulated in the presence of MG132 would immediately be dephosphorylated in response to heat. Thus, we pretreated DREB2A FL-overexpressing plants with MG132 for 2 h and then added an amount of cycloheximide (CHX) sufficient to halt de novo synthesis of the DREB2A protein for 30 min before heat stress (Fig. 9D). In samples without CHX, a significant amount of nonphosphorylated DREB2A accumulated after 2 h of the heat-stress treatment, regardless of MG132 treatment (Fig. 9, D and E). In contrast, only a small amount of nonphosphorylated DREB2A appeared after heat stress in the presence of both MG132 and CHX, whereas most of the DREB2A protein remained phosphorylated (Fig. 9, D and E). This result suggests that even though a net dephosphorylation of DREB2A occurs under heat stress, the main cause of the reduction in the phosphorylation level of DREB2A in response to heat is the reduced phosphorylation rate of de novo synthesized DREB2A.

Discussion

We previously reported that the deletion of the NRD increases the stability of the DREB2A protein and activates the expression of downstream genes (8, 9). We also reported that DREB2A is stabilized in response to heat and that the level of
DREB2A is a determinant of the downstream gene expression level (9, 11). Therefore, it has been hypothesized that in response to stress, the function of the NRD is prevented, which stabilizes and activates DREB2A, although the mechanism is unknown. Here, we report that the NRD contains major phosphorylation sites, and the phosphorylation of the NRD in turn facilitates the degradation of DREB2A (Figs. 4 and 8). In addition, the phosphorylation level of DREB2A decreases in response to heat in a temperature-dependent manner, whereas DREB2A is stabilized (Fig. 9). Collectively, our results suggest that regulation via phosphorylation of the NRD is a mechanism that ensures rapid degradation of DREB2A when the protein is not required but increases the stability of DREB2A in response to heat to activate downstream gene expression and improve thermotolerance (Fig. 10). This study showed that the NRD acts as a temperature-sensitive degron and functions as an actual target of temperature-dependent post-translational regulation of DREB2A.

Destabilization of proteins by phosphorylation is often based on the phosphorylation-enhanced or phosphorylation-dependent formation of specific ubiquitin ligase-substrate complexes in animals, yeast, and plants (28, 29). Two types of DREB2A-interacting proteins, DRIPs and BPMs, have been shown to facilitate the degradation of DREB2A via the ubiquitin-proteasome system, although their function in the stabilization of DREB2A in response to heat remains unclear (10, 12). Among these DREB2A-interacting proteins, BPMs, which are homologs of the mammalian speckle-type pox virus and zinc finger protein (SPOP), which functions as an adaptor subunit of a CUL3-based E3 ligase complex, interact with the...
N-NRD and mediate NRD-dependent degradation (12). BPM2, which is one of six *A. thaliana* BPMs, recognizes the SPOP-binding consensus–like motif, /H9278/H9266SXT(S/T) (where /H9278 indicates nonpolar, /H9266 indicates polar, and X is any amino acid), which corresponds to the VTSTS (Val142–Ser146) sequence in the NRD (12). This motif overlaps with the core S/T cluster (Thr143–Ser147), and multiple Ala mutations (VTAAA) disrupt the interaction between BPM2 and the NRD (12), indicating that the core S/T cluster functions as a BPM-binding site. Therefore, the most likely hypothesis is that phosphorylation of the NRD enhances the interaction between BPM2 and DREB2A to facilitate the degradation of DREB2A.

BPMs seem to provide general degradation machinery, because their involvement in various biological processes entails targeting various transcription factors such as related to *apetala 2.4* (30), homeobox protein 6 (31), wrinkled 1 (32), and MYB56 (33). Here, we report that modification of the BPM-binding site in DREB2A changes in response to stress. It is intriguing to hypothesize that the stress-dependent change in phosphorylation may act as a switch enabling the specific escape of DREB2A from BPM-mediated degradation. Investigation of the effects of NRD phosphorylation on the interaction with BPMs, as well as the identification of other phosphorylation-dependent molecules of DREB2A, will be necessary to

**Figure 9. The phosphorylation level of DREB2A is reduced in response to heat.** A, changes in the phosphorylation level of DREB2A in response to heat. Transgenic plants expressing DREB2A FL (line a) were pretreated either without or with MG132 and exposed to heat stress, and the accumulation and phosphorylation levels of DREB2A were analyzed using SDS-PAGE and Phos-tag PAGE gels. B, densitometric scan data of the Phos-tag PAGE gel images shown in A. C, phosphorylation level and stability of DREB2A in response to different degrees of heat stress. Transgenic plants expressing DREB2A FL (line a) were pretreated either without or with MG132 and then without or with CHX. After 2 h of heat stress, the protein was extracted, and the accumulation and phosphorylation levels of DREB2A were analyzed using SDS-PAGE and Phos-tag PAGE gels. All data represent the results from two independent experiments. D, analysis of dephosphorylation in response to heat stress. Transgenic plants expressing DREB2A FL (line a) were pretreated either without or with MG132 and then without or with CHX. After 2 h of heat stress, the protein was extracted, and the accumulation and phosphorylation levels of DREB2A were analyzed using SDS-PAGE and Phos-tag PAGE gels. All data represent the results from two independent experiments. E, densitometric scan data of the Phos-tag PAGE gel image shown in D. The data obtained from DREB2A FL plants that were treated for 2 h at either 37 °C or room temperature (RT) in the presence of MG132 are shown.
Regulation of DREB2A by phosphorylation

![Figure 10. Proposed model for the role of NRD phosphorylation in the stability regulation of DREB2A. Under nonstress conditions in which DREB2A is unnecessary, nascent DREB2A is immediately phosphorylated at multiple Ser/Thr residues in the NRD, probably by CK1. The phosphorylation of the NRD facilitates the degradation of DREB2A, keeping the level of DREB2A low. In response to heat stress, this phosphorylation of nascent DREB2A is inhibited, which inactivates the phosphorylation-facilitated degradation mechanism. The stabilization of DREB2A in response to heat allows its accumulation to activate the expression of stress-inducible genes.

clarify the molecular basis of the phosphorylation-facilitated degradation of DREB2A.

In addition to the interaction with E3, phosphorylation of the NRD may also be involved in the maintenance of disordered structure to ensure proteasomal proteolysis. Disordered structure near a polyubiquitin signal serves as an initiation site that is necessary for proteasomal proteolysis (34, 35). In the case of the bovine papilloma virus E2 protein, a well-characterized short-lived transcription factor, phosphorylation of two Ser residues in an acidic hinge region between the transactivation and DNA-lived transcription factor, phosphorylation of two Ser residues involved. Phenomena will be clarified by determining the actual phosphorylation sites in these mutants. In the future, the reason for these might be due to the replacement of largely different phosphor-

We found that Ser/Thr cluster(s) were enriched in transcription factors (Fig. 1, B and C). Although the consequence of phosphorylation may differ among the proteins, this finding increases the possibility that these Ser/Thr clusters function as phosphorylation sites. The results of inhibitor experiments in vitro (Fig. 6B) and in vivo (Fig. 8) suggest that CK1 may act as a major kinase in the phosphorylation of the NRD. CK1 is a ubiquitous acidophilic kinase that often mediates multiple phosphorylations via progressive phosphorylation (39). Phos-tag PAGE analyses of the mutant DREB2A proteins suggested multiple phosphorylations in the NRD (Fig. 4 and Fig. S2). Furthermore, partial mutation of Ser/Thr residues to an acidic residue (Asp) in the NRD facilitated the phosphorylation of other sites, whereas Ala mutations at the same residues did not (Figs. 4 and Fig. S2). These results agree with the known nature of CK1, and progressive phosphorylation may therefore play a role in the phosphorylation of DREB2A.

In addition to the NRD, both the N-terminal (1–135) and C-terminal (166–335) fragments were phosphorylated in vitro (Fig. 6B). Notably, strong heparin-sensitive kinase activity toward the C-terminal fragment was detected in vitro (Fig. 6B). However, the contribution of phosphorylation outside the NRD seems to be minor in vivo because a large proportion of DREB2A CA remains unphosphorylated (Fig. 4, B and C). The residual phosphorylation in DREB2A CA seems to also be mediated by CK1 because this phosphorylation was diminished by the CK1 inhibitor treatment (Fig. 8). CK1 inhibitor treatment, as well as proteasome inhibitor treatment, slightly increased the protein level of DREB2A CA (Fig. 8, A and C), suggesting that the residual phosphorylation of the C-terminal region also contributed to the destabilization of DREB2A, although its contribution seems to be minor. On the other hand, the phosphorylation of the N-terminal region appears to be rare. Therefore, the regulation of DREB2A stability by DRIPs, which recognize the N-terminal region (10, 12), is assumed to be independent of the phosphorylation of DREB2A.

CK1 functions in various plant biological processes, such as flowering (40, 41), light signaling (42), hormone biosynthesis and signaling (43, 44), and organization of the cytoskeleton (45, 46). With regard to the regulation of stress responses, one report has shown the involvement of CK1 in the osmotic-stress-induced phosphorylation of histone H3, which may positively regulate drought tolerance (47). There are 17 CK1 and CK1-like genes in A. thaliana, and a subset of these genes encode nuclear-localized CK1s (42, 47, 48). These nuclear-localized CK1s are candidates for DREB2A-phosphorylating kinases because the phosphorylation of DREB2A seems to occur in the nucleus (Fig. 5). The identification of the responsible CK1s will be important to confirm the involvement of CK1 proteins in the phosphorylation and regulation of DREB2A.
Regulation of DREB2A by phosphorylation

Here, we showed that the phosphorylation level of DREB2A is reduced in response to heat. Reduced phosphorylation in response to heat has been reported for the ribosomal protein S6 (18) and the transcription factor NAC019 (19); the former is due to the heat sensitivity of S6 kinase (49), and the latter appears to depend on a C-terminal domain phosphatase (19). Our results suggest that a reduction in the phosphorylation rate rather than an elevation of phosphatase activity mainly accounts for the reduced phosphorylation levels of DREB2A in response to heat (Fig. 9D). Therefore, the reduction in the phosphorylation level of DREB2A does not seem to be related to the previously described heat-induced dephosphorylation events.

In mammals, intracellular localization and complex formation, which can be regulated by interactions with scaffold proteins, are important factors that control the access of CK1 to its specific substrates (39). Although the direct involvement of CK1 in the phosphorylation of DREB2A remains to be proven, the specific inhibition of CK1 activity toward DREB2A, either by reducing interactions with scaffold proteins or by increasing interactions with inhibitory proteins to sequester CK1, is the most likely explanation for the regulation of DREB2A phosphorylation. The future identification of the CK1s responsible for DREB2A phosphorylation, and any specific molecules that may inhibit or enhance the conditional interaction between DREB2A and CK1, as well as analyses of their interactions in response to heat, will be important to the mechanism for the heat-induced reduction in DREB2A phosphorylation, which will then provide a way to identify the heat-sensory system that leads to DREB2A-activated transcription.

This study showed the involvement of phosphorylation in the post-translational regulation of the stability of DREB2A. DREB2A is also regulated at the transcriptional level, and the expression of the DREB2A gene is activated in response to various external and internal environmental cues such as heat, osmotic stress, and reactive oxygen species (7, 9, 50–53). Under stress-prone and fluctuating natural conditions, an excess of DREB2A transcripts can result, either from high background levels of DREB2A transcription or from the relief of the stress conditions. It is important to rapidly degrade unnecessary DREB2A that is translated from excess DREB2A transcripts, because overaccumulation of DREB2A negatively affects plant growth. Thus, the regulation of DREB2A stability by changing its phosphorylation status to rapidly and finely control the amount of DREB2A may help plants cope with environmental fluctuations under complex stress conditions by balancing stress tolerance and growth. The rapid and proper post-translational regulatory mechanism of DREB2A may provide clues to understanding plant responses to the complex stress conditions that occur in natural environments.

Experimental procedures

Plant materials and growth conditions

A. thaliana (L.) Heynh. ecotype Columbia seedlings were grown on germination medium agar plates for 2 weeks at 22 °C under a day/night 16-h photoperiod at a photon flux density of 40 μmol m⁻² s⁻¹, as described previously (54). Transgenic A. thaliana plants were generated in the dreb2a-1 (9) background using the plasmids described in the supporting text. Homozygous lines of the T3 generation that carry a single locus insertion and show similar expression levels were selected as described in the supporting text and used for experiments.

Stress and chemical treatments of seedlings

For the chemical treatment, 8–12 seedlings were picked from the growth medium and transferred to chemical solutions in the wells of an assay plate. The concentrations of the chemicals were 100 μM for MG132, 200 μM for PF-670462 and DRB (TCI, Tokyo, Japan), and 0.2 mg/ml for CHX. The duration of the MG132 treatment was 2.5 h for seedlings and 1 h for protoplasts, unless otherwise specified. Heat stress was applied to the seedlings by transferring the agar or assay plates into a hybridization oven set at the desired temperature.

Thermotolerance test

Thermotolerance testing of the seedlings was performed as described previously (55), with modifications. A heat shock treatment (42 °C for 80 min) was applied to 10-day-old seedlings, which then recovered for 7 days. Total chlorophyll was extracted by immersing the seedlings in 80% (v/v) acetone, and the chlorophyll content was determined using a spectrometric method (56).

Transient expression assays in protoplasts

Transient expression assays using protoplasts derived from A. thaliana mesophyll cells were performed as previously described (57). Effector plasmids were constructed as described in the supporting text. The reporter and internal control plasmids used have been described previously (7). For the preparation of samples for immunoblot analysis, pGKX-NsGFP was used as the internal control plasmid (10).

Immunoblot analysis

Immunoblot analyses using conventional SDS-PAGE were performed as previously described with samples prepared from protoplasts (20) and seedlings (11). Phosphorylation levels were analyzed by Zn²⁺-Phos-tag PAGE using 6.5% polyacrylamide gels containing 50 μM Phos-tag acrylamide (Wako, Osaka, Japan) (21). The anti-DREB2A antibodies (11) and anti-GFP antibodies (58) used were rabbit polyclonal antibodies that were raised against the 166–335 fragment of DREB2A and full-length GFP, respectively. Mouse anti-histone H3 antibody was obtained from Abcam (ab17911). Protein bands were visualized by chemiluminescence using ECL Prime (GE Healthcare) and detected by an image analyzer (LAS4000, GE Healthcare). Denitometric scanning of the original 16-bit data were performed using ImageJ software.

A protein phosphatase treatment of the protein samples

Protein samples that had been prepared for immunoblot analysis were subjected to TCA precipitation. After precipitation, the pellets were washed twice with acetone, dried, dissolved in 0.8 initial volumes of solubilization buffer (8 M urea, 50 mM HEPES-KOH, pH 7.5, 100 mM NaCl, 2 mM DTT, 10% (v/v) glycerol, 1% (v/v) Triton X-100), and dialyzed against solubilization buffer without urea. The phosphatase reaction mix (25


μl) contained 19 μl of the dialyzed protein sample, 1× NEBuffer Pack for Protein MetalloPhosphatases (New England Biolabs), 1 mM MnCl₂, 1× cComplete protease inhibitor mixture (−EDTA) (Roche), and 1 μl (400 units) of λ protein phosphatase (New England Biolabs). After incubation at 30 °C for 30 min, the reaction was stopped by adding 8 μl of 4× SDS-PAGE sample buffer and boiling at 95 °C for 3 min.

Expression analysis

RNA was extracted from seedlings grown under previously described conditions (58). Transcripts were quantified using quantitative RT-PCR with standard curves. ACT2 was used as an internal standard. The primers used for quantitative RT-PCR are listed in Table S1.

In vitro kinase assays

In vitro kinase assays were performed as described (59). Each reaction contained 50 mM HEPES-KOH (pH 7.5), 10 mM MgCl₂, 0.5 mM CaCl₂, 1 mM DTT, 1 mM MnCl₂, 1 μM nuclear protein, 0.3 μg of substrate, and 10 mM [γ-32P]ATP (150 kBq) in a volume of 15 μl. Each reaction was associated with a parallel reaction using cold ATP for immunoblot analyses. The preparation of nuclear protein from A. thaliana seedlings, the construction of the expression plasmids, the expression in E. coli, and the purification of the recombinant proteins are described in the supporting text. The final concentrations of the protein kinase inhibitors were 10 μM staurosporine, 10 μM bikini, and 100 μg/ml heparin. For immunoblot analyses, an anti-GST antibody (PM013-7; MBL, Nagoya, Japan) was used.

Determination of phosphorylation sites by MS

In-gel digestion of in vitro phosphorylated recombinant proteins was performed as previously described (12) with modifications. The bands corresponding to recombinant proteins were excised, and the peptides were prepared by in-gel digestion with Glu-C (20 ng/μl, 50 μl/gel slice; Promega) or sequential double digestion with Trypsin/Lys-C mix (20 ng/μl, 50 μl/gel slice; Promega) and Glu-C. The recovered peptides were desalted using GL-Tip SDB and GL-Tip GC tip columns (GL Sciences, Tokyo, Japan) according to the manufacturer’s instructions.

Mass spectrometric analysis was performed using Trip-leTOF 5600 (SCIEX) with an Autosampler-2 1D plus (Eksigent). Each sample was isolated using a MonoCap C18 high resolution 2000 (2000-mm × 0.1-mm inside diameter, 2-μm particle size; GL Sciences). The sample was concentrated in the analytical column using 0.1% formic acid solution at a flow rate of 500 nl/min over 30 min. The mobile phase comprised 2% acetonitrile and 0.1% formic acid (A) and 80% acetonitrile and 0.1% formic acid (B), and the flow rate was 500 nl/min. A linear gradient of A:B = 98:2 to A:B = 60:40 in 300 min, A:B = 10:90 in 20 min, and A:B = 98:2 in 40 min was used in this analysis. The MS scan range was a mass/charge ratio (m/z) of 400–1250, and the top 20 precursor ions were selected for subsequent MS/MS scans in high-sensitivity mode. The MS/MS data were analyzed using ProteinPilot 5.0 software (SCIEX) and subsequently annotated using a custom database of GST-DREB2A 136–335 for peptide identification.

Regulation of DREB2A by phosphorylation

Sequences of TAIR10 proteins were obtained from the Arabidopsis Information Resource. Representative peptides for each nuclear locus were used for further analyses. Transcription factors were defined according to AtTFDB (AGRIS, http://agris-knowledgebase.org/AtTFDB/) (60). Ser/Thr clusters ([S/T]ₙ where n ≥ 5) were identified by using the gregexpr function of R (version 3.3.2) with the regular expression [ST][ST][S/T][S/T][S/T][S/T][S/T][S/T]. GO enrichment analysis was conducted according to the GO slim annotations available in the Arabidopsis Information Resource.

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