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Identification of the amino acids crucial for the activities of drought responsive element binding factors (DREBs) of *Brassica napus*

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Abstract **We have previously identified two homologous groups of BnDREBs in *Brassica napus*, the *trans*-active BnDREBI and the *trans*-inactive BnDREBII, which provided an ideal system to study the *trans*-activation of DREB1/CBF. Deletion analysis indicated that the two additional regions in BnDREBI contributed little to the transcriptional activity. Domain swapping analysis indicated that all the domains contributed to the activity of BnDREBI, including the ERF/AP2 DNA binding domain. Through site-directed mutagenesis, we identified nine residues that were involved in the activity of BnDREBI, among which six residues are specific to BnDREBI, and three are common to DREB1A.**

Keywords: DRE; DREB1/CBF; *Trans*-activation; Transcriptional factors; *Brassica napus*

1. Introduction

During the course of growth and development, plants might be exposed to various abiotic stresses, such as cold, drought and high salinity. Under these conditions, a number of signal pathways will be activated, which results in the transcriptional reprogramming to bestow plants with the resistance to these stresses [1–5]. Many transcriptional factors involved in these stress-resistance pathways have been identified, among which the drought responsive element binding factors (DREBs)/C-repeat binding factors (CBF) are one of the most important factors [6–8]. The DREBs/CBFs have been shown to mediate the expression of various stress-inducible genes, and they play a significant role in plant stress tolerance [6–10]. Over-expression of *Arabidopsis* DREB1A (AtDREB1) or CBF1 greatly increased the tolerance to freezing, high salinity and drought in transgenic *Arabidopsis* [7,11,12], and over-expression of the constitutive active AtDREB2A greatly enhanced drought stress tolerance in *Arabidopsis* [13].

The plant specific ERF/AP2 DNA-binding domain of DREBs have been well characterized, and it is also quite conserved in ethylene responsive element binding proteins (EREBPs) [14,15]. The ERF/AP2 domain of AtERF1, a member of EREBPs family, consists of a three-stranded anti-parallel β-sheet and an α-helix packed approximately parallel to the β-sheet [16]. Arginine and tryptophan residues in the β-sheet are identified to recognize the GCC-box element [16]. The ERF/AP2 domain of DREBs is highly homologous with that of EREBPs, but the key amino acids for binding of the *cis*-elements are different [17]. Val14 and Glu19, especially Val14, have been demonstrated to be essential for specific binding with drought responsive element (DRE) [17,18]. There are also some proteins that were reported to be able to bind with both GCC-box and DRE, such as AtDREB2A [17], TINY2 [19], Tsi1[20] and BnDREBIII [21]. Our recent studies found that Ala37 in the ERF/AP2 domain plays a crucial role in binding both DRE and GCC-box [21].

However, little is known about the activity of the special family to activate transcription. The C-terminal 98 amino acids were essential for the activity of CBF1, and four hydrophobic motifs were identified to function positively, but redundantly [22]. The essential domain in the activity of AtDREB2A was located between residues 254–335, and there was a negative regulatory domain located between the residues 136–165 [13]. By far, there is no report about the amino acids that are crucial in the activity of DREB1/CBFs.

In a previous paper, we have reported two highly homologous groups of BnDREBs, which had similar DNA binding abilities, but completely different activities to activate transcription: BnDREBI is *trans*-active, while BnDREBII is *trans*-inactive [23]. Then the two groups of DREBs provide a favorable system to study the activities of DREB1/CBFs. Here, through domain swapping analysis, we found that replacing any of the domains, even the ERF/AP2 domain, of BnDREBII with the counterpart in BnDREBI could generate an active BnDREBII. And we found that nine amino acids were responsible for the difference in the activities of these two groups of BnDREBs. The findings here provided some useful information for understanding the *trans*-activation mechanism of DREB1/CBF.

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2. Materials and methods

2.1. Materials

BnDREBI-5 and *BnDREBII-1* were cloned in our previous report [23]. 3-AT and ONPG were from Sigma, St. Louis, USA. Amino acids dropout was from Clontech, Palo Alto, USA. T_4 DNA ligase was from Promega, Madison, USA. x-β-gal was from TaKaRa, Tokyo, China. All the other chemicals were local products of analytical grade.

2.2. Deletion and chimera constructs

BnDREBI-5 and *BnDREBII-1* were constructed into the yeast expression vector, YepGAP [7], as described previously [23]. Two single deletion mutants of BnDREBI-5, I-5ΔC2 and I-5ΔC4, and a double deletion mutants, I-5ΔC2C4, were constructed, as illustrated in Fig. 2A. Seven chimeras of BnDREBI-5 and BnDREBII-1 were constructed, INIIC, IINIC, II-1SN, II-1SAP2, II-1SC1, II-1SC1C3, and II-1SC1C3C5, as illustrated in Fig. 2C. The deletions and chimeras were constructed by PCR-mediated overlapping using two subsequent PCR amplifications [24,25], and all the mutants were sequenced and cloned into YepGAP. The primers used for the constructs can be supplied as requested.

2.3. Site-directed mutagenesis

According to the differences of BnDREBII with BnDREBI or AtDREB1A (Fig. 1, Table 1), 19 site-directed mutagenesis of BnDREBII-1 were constructed to identify the key amino acids for *trans*-activation. The site-directed mutants were generated by PCR-mediated overlapping [24,25], and the mutants were sequenced and cloned into YepGAP for yeast one hybrid assay. The primers used for site-directed mutagenesis can be supplied as requested.

2.4. Yeast one-hybrid and β-galactosidase activity assay

To detect the mutations on the *trans*-activation activity, all the constructs were transformed into the yeasts harboring wild-type DRE or mutated DRE with *HIS3* and *lacZ* as the reporter genes, as was described previously [7]. The transformants were cultured on SD/-His/-Ura/-Trp plates with or without 30 mM 3-amino-1,2,4-triazole (3-AT). When the yeasts were larger than 1 mm, three clones of each transformants were cultured in liquid SD/-His/-Ura/-Trp medium to grow to OD600 > 1.0, and quantitative analysis of β-galactosidase activity was carried out, using *o*-nitrophenyl *p*-D-galactopyranoside (ONPG) as a substrate according to the Yeast Protocols Handbook (Clontech). Colony-lift filter assay was also carried out to give a qualitative assay of the *trans*-activation activity of the constructs.

3. Results

As was shown in our previous studies, the *trans*-active BnDREBI and the trans-inactive BnDREBII were highly homologous except that BnDREBI had two additional

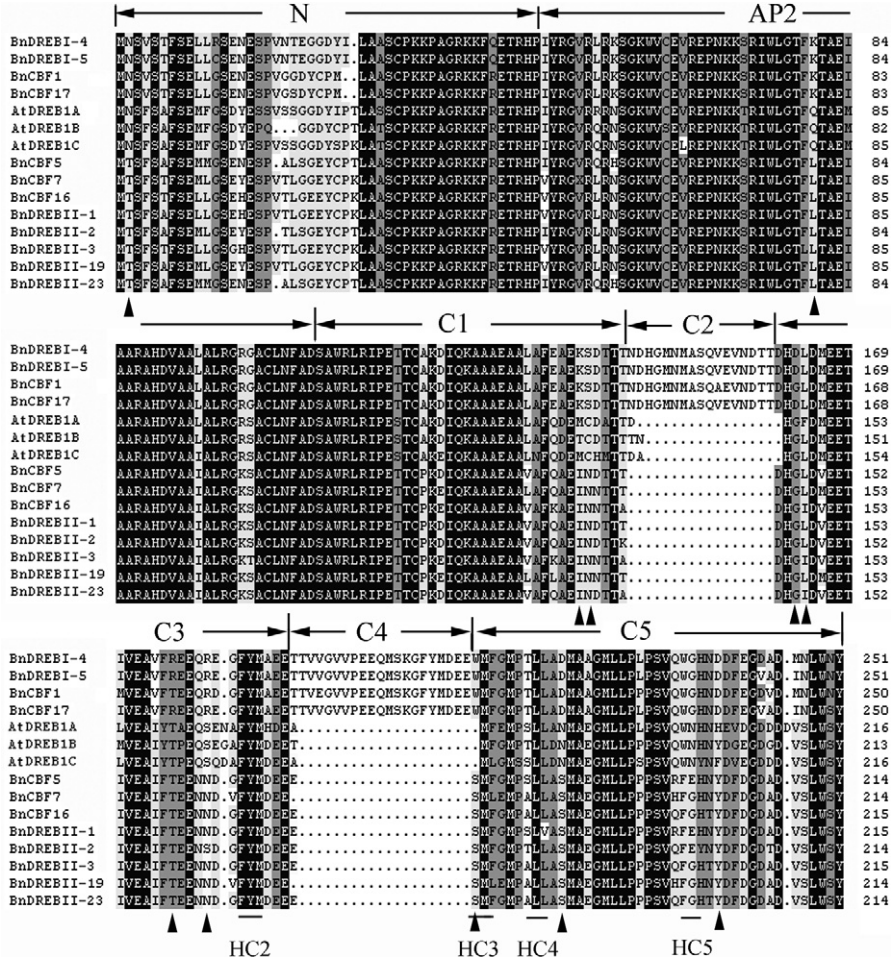

Fig. 1. Sequence alignment and subdivision of the two groups of BnDREBs and AtDREB1A-C. The accession numbers of these DREBs are: BnCBF16 (AF499033), BnDREBII-3 (AY444875), BnDREBII-1 (AY437878), BnCBF7 (AF499032), BnDREBII-19 (AY444877), BnDREBII-2 (AY444874), BnDREBII-3 (AY444875), BnCBF5 (AF499031), BnDREBII-23 (AY444876), BnCBF17 (AF499034), BnCBF1 (AAL38242), BnDREBI-4 (EF625342), BnDREBI-5 (EF625343), AtDREB1A (AB007787), DREB1B (AB007788), and DREBIC (AB007789). N, AP2, C1, C2, C3, C4 and C5 are the regions. HC2, HC3, HC4 and HC5 are the four hydrophobic motifs identified previously [22]. The amino acids different in BnDREBI, BnDREBII and AtDREB1A-C are indicated by closed triangles.

Table 1
The differences in amino acids of BnDREBII-1, BnDREBI-5 and AtDREB1A

Position[a]	Amino acid		
	BnDREBII-1	BnDREBI-5	AtDREB1A
2	T	N	N
81	L	K	Q
139–140	IN	KS	MC
147–148	GL	DL	GF
160–164	TEENN	REEQR	TAEQS
174	S	W	–[b]
184	S	D	N
202	Y	D	H

[a]Numbering according to BnDREBII-1.
[b]There is no amino acid in the corresponding position in AtDREB1A.

fragments in its C-terminal [23]. To further identify the regions that might be responsible for the activity of DREBs to activate transcription, a sequence alignment was made among the two groups of BnDREBs, the reported BnCBFs [26,27] and AtDREB1A-C [6,7,28] (Fig. 1), among which BnCBF1 [26] and BnCBF17 [27] are quite similar to BnDREBI, and BnCBF5/7/16 [27] to BnDREBII. The two groups of BnDREBs were also highly homologous to AtDREB1A-C. To facilitate the identification of the regions crucial for the activities of the two groups of BnDREBs, the full length of these BnDREBs were subdivided into seven regions, N, AP2, C1, C2, C3, C4 and C5, among which C2 and C4 are specific to BnDREBI (Fig. 1). The amino acids different among BnDREBII-1, BnDREBI-5 and AtDREB1A were listed in Table 1.

Since the most obvious difference between these two groups of DREBs was that BnDREBI had two additional C-terminal fragments, C2 and C4, it was meaningful to explore whether the difference in the activities of the BnDREBs was due to these two fragments. Three deletion mutants including two single deletions, I-5ΔC2 and I-5ΔC4, and a double deletion I-5ΔC2C4, were constructed, and the activities of these deletion mutants were tested by yeast one-hybrid and β-galactosidase activity assay (Fig. 2). Surprisingly, I-5Δ C2 and I-5ΔC4 showed only a slight decrease in the activity, and I-5ΔC2C4 had almost the same activity as BnDREBI-5 (Fig. 2A). As shown in Fig. 2B, all the deletion mutants grew well on SD/-His/-Ura/-Trp medium with 30 mM 3-AT and activated the expression of the downstream *HIS3* and *LacZ*. The control, DREBII-1, could not activate the expression of β-galactosidase. The results here indicated that the two additional C-terminal fragments were not essential for the activity of BnDREBI-5.

Then which regions were responsible for the activity of BnDREBI to activate transcription? To further investigate whether the N- or C-terminal of BnDREBI-5 contained the information for the activity, two chimeras, INIIC and IINIC, were constructed through domain swapping. INIIC was the fusion protein of the N-terminal of BnDREI-5 (N and AP2) with the C-terminal of BnDREBII-1 (C1, C3 and C5), and IINIC was the fusion of the N-terminal of BnDREBII-1 (N and AP2) and the C-terminal of BnDREBI-5 (C1, C2, C3, C4 and C5), as illustrated in Fig. 2C. Surprisingly, both INIIC and IINIC grew well on SD/-His/-Ura/-Trp containing 30 mM 3-AT and activated the expression of the downstream β-galactosidase. The activities of IINIC and INIIC were 93.7% and 69.7% of BnDREBI-5, respectively (Fig. 2C). The results here indicated that both the N- and C-terminals of BnDREBI-5 participated in the activity of BnDREBI-5 to activate transcription, and that the C-terminal might contribute more.

A more detailed study was achieved by the construction of five more chimeras, II-1SN, II-1SAP2, II-1SC1, II-1SC1C3 and II-1SC1C3C5, which were constructed by substitution of the N, AP2, C1, C1C3 and C1C3C5 regions of BnDREBII-1 by their counterparts in BnDREBI-5 (Fig. 2C). Surprisingly, all the five chimeras showed the activities though to different extents, which indicated that all these regions were involved in the activity of BnDREBI to activate transcription.

To further determine which amino acids are essential in each region, a series of site-directed mutants of BnDREBII-1 were constructed according to the results of sequence alignment of the two groups of BnDREBs and AtDREB1A-C (Fig. 1, Table 1). The major difference in the N-region was the 2nd amino acid, which is Thr in BnDREBII, but Asn in BnDREBI and AtDREB1A. Therefore, a site-directed mutant of BnDREBII-1, II-1T2 N, was constructed to explore whether this amino acid is essential for trans-activation in the N-region. Unlike BnDREBII-1, II-1T2N activated the expression of downstream genes *HIS3* and *lacZ* in yeasts (Fig. 3). The activity of II-1T2N was 37.3% of that of BnDREBI-5, almost identical to that of II-1SN. Then Asn2 might be the key amino acid in the N-region. As it is Asn in the *trans*-active BnDREBI and AtDREB1A-C, it might be common in DREB1/CBF that Asn in this site was responsible for the activity.

One of the most surprising things is that the ERF/AP2 domain of BnDREBI-5 was also involved in the activity transcription (Fig. 2C). As shown in Table 1, the difference among BnDREBI-5, BnDREBII-1 and AtDREB1A in the ERF/AP2 domain was that it was Leu81 in BnDREBII-1, while Lys80 in BnDREBI-5 and Gln81 in AtDREB1A. Then two site-directed mutants II-1L81K and II-1L81Q were constructed and analyzed by yeast one-hybrid and β-galactosidase activity assay. As was shown in Fig. 3, II-1L81K could activate the expression of the downstream genes *HIS3* and *lacZ*, and its activity was 28.5% of that of BnDREBI-5, similar to II-1SAP2. However, II-1L81Q could neither activate the expression of *HIS3* and *lacZ*, nor show any *trans*-activation activity (Fig. 3). Then the results here indicated that Lys80 was the key amino acid in the ERF/AP2 domain, which seemed to be specific to BnDREBI.

The major difference in the C1 region was that it was Ile139Asn140 in BnDREBII-1, while it was Lys138 Ser139 in BnDREBI-5, and Met139Cys140 in AtDREB1A (Table 1). To explore the role of this small motif in the activity, two double mutants, II-1IN-KS and II-1IN-MC, were constructed and analyzed by yeast one-hybrid and β-galactosidase activity assay. As was shown in Fig. 4, II-1IN-KS activated the downstream *HIS3* and *LacZ*, and showed the activity of 36.1% of BnDREBI-5, but II-1IN-MC was *trans*-inactive. The results here suggested that this small motif, Lys138Ser139, was responsible for the activity of the C1 region in BnDREBI-5. To determine whether Lys138 or Ser139 is essential for the activity, two single-site mutants of BnDREBII-1, II-1I139K and II-1N140S, were constructed. Yeast one-hybrid and β-galactosidase activity assay showed that II-1I139K was *trans*-inactive, while II-1N140S was *trans*-active, which suggested that Ser139 in BnDREBI-5 was more important in this motif (Fig. 4). However, it should be noted that the activity of II-1N140S (14.3%), although *trans*-active, was much lower than that of

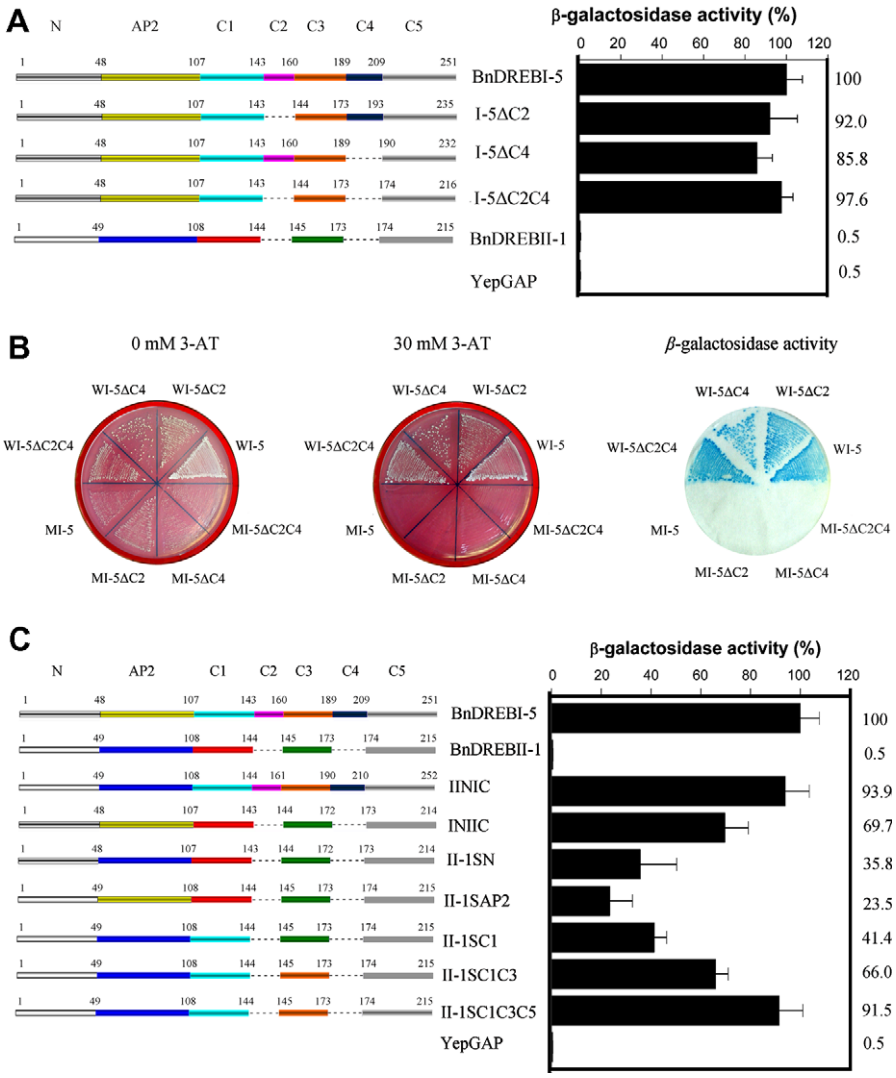

Fig. 2. Identification of the *trans*-active regions in BnDREBI-5. (A) β-Galactosidase activity analysis of the deletion mutants of BnDREBI-5. (B) Yeast one-hybrid analysis of the *trans*-activation ability of the deletion mutants of BnDREBI-5. (C) Yeast one-hybrid (left) and β-galactosidase activity (right) analysis of the chimeras between BnDREBI-5 and BnDREBII-1. The *trans*-activation activity of BnDREBI-5 was taken as 100%. The constructs were transformed into yeasts containing wild-type DRE (WDRE) or mutated DRE (MDRE) for yeast one-hybrid assay. The transformants were cultured on SD/-His/-Ura/-Trp with or without 30 mM 3-AT, and used for colony-lift filter assay. W and M in the beginning of each name indicates the transformants that harbored WDRE and MDRE, respectively.

II-1IN-KS (36.1%). Then it can be concluded that Ser139 was enough to bestow the C1 region with the activity, but Lys138, though not sufficient for the activity, was also needed to exhibit its maximum activity. The possible role of Lys138 might contribute to stabilize the conformation of the activation domain.

C3 region contains two motifs that are different between the DREBs analyzed here (Fig. 1). One motif from 160 to 164 is TEENN in BnDREBII-1, while it is REEQR (176–180) in BnDREBI-5 and TAEQS (160–164) in AtDREB1A. Based on this, TEENN was mutated to REEQR and TAEQS to explore whether this motif played an essential role in the activity. As was shown in Fig. 4, II-1REEQR showed 51% of the activity of BnDREBI-5, while II-1TAEQS was *trans*-inactive. Comparing the motifs in the three DREBs, we can deduce that the two Arg residues in REEQR might be essential in this motif. Then two site-directed mutants, II-1T160R and II-1N164R, were constructed to confirm the hypothesis above. Yeast one-hybrid and β-galactosidase activity assay showed that II-1T160R and II-1N164R had similar abilities to activate the expression of the downstream genes, about half of II-1REEQR (Fig. 4). Therefore, Arg176 and Arg180 were the key amino acids in the REEQR motif.

It can also be observed that the 147th amino acid is Gly in BnDREBII-1 and AtDREB1A, but it is Asp163 in BnDREBI-5. The 148th amino acid is Phe in AtDREB1A, but the counterpart was Leu in BnDREBI-5 and BnDREBII-1. Two single mutants, II-1G147D and II-1L148F, and a double mutant, II-1GL-DF, were constructed to explore whether these two amino acids are essential. Interestingly, all the mutants can activate the expression of the downstream genes, and the activity of II-1GL-DF was near to the sum of that of II-1G147D and II-1L148F (Fig. 4). The results here indicated that Asp163, specific to BnDREBI-5, and Phe148, specific to AtDREB1A, were the key amino acids in the C3 region.

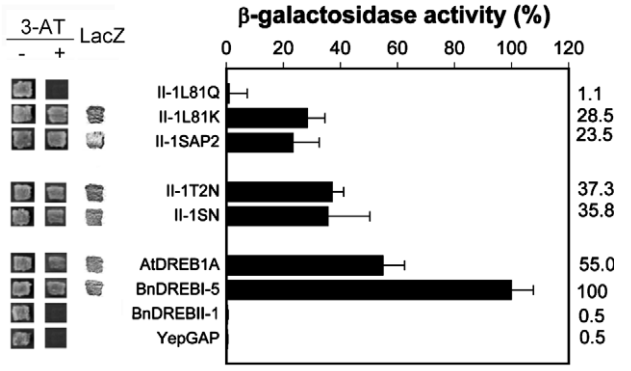

Fig. 3. Identification of the essential amino acids in the N-terminal of BnDREBI-5 by yeast one-hybrid (left) and β-galactosidase activity (right) assay. The mutants were transformed into yeast harboring WDRE. The transformants were cultured on SD/-His/-Ura/-Trp with (+) or without (−) 30 mM 3-AT, and were analyzed by colony-lift filter assay (LacZ).

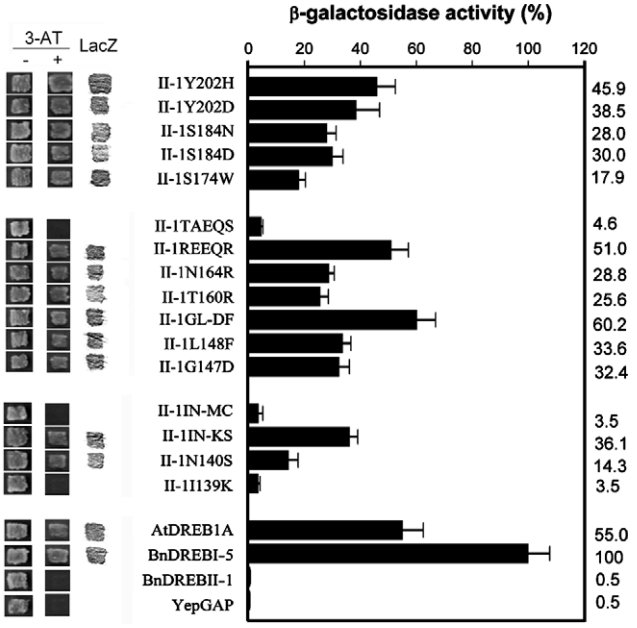

Fig. 4. Identification of the key amino acid responsible for *trans*-activation in the C-terminal of BnDREBI-5 using yeast one-hybrid (left) and β-galactosidase activity (right) assay.

Sequence alignment in the C5 region identified three residues (residues 174, 184, and 202 in BnDREBII-1) that were quite different in this region (Table 1). The first one is Ser174 in BDREBII, Trp211 in BnDREBI, while no counterpart in AtDREB1A. As was shown in Fig. 4, mutation of Ser174 in BnDREBII to Trp, the counterpart in BnDREBI, generated a *trans*-active form of BnDREBII-1, II-1S174W, with the activity about 17.9% of that of BnDREBI-5. Then BnDREBI-specific Trp211 was the key amino acid in C5 region.

The second is Ser184 in BDREBII, Asp221 in BnDREBI, and Asn184 in AtDREB1A. Interestingly, II-1S184D and II-1S184N had similar abilities to activate the downstream genes (Fig. 4). Then either Asp or Asn was enough to show *trans*-active activity, which might be common in DREB1/CBF1.

The third is Tyr202 in BDREBII, Asp239 in BnDREBI, and His202 in AtDREB1A. Similarly, II-1Y202D and II-1Y202H could also activate the expression of downstream genes (Fig. 4), indicating both Asp and His were the essential amino acids in this site, which might also be common in DREB1/CBF1.

4. Discussion

Although numerous papers have been published on DREBs or CBFs since it was first cloned [6,7], there are only few reports on the characterization of the crucial domains in the activity to activate transcription. Because there was redundancy in the activity of the DREB1/CBF family [22], it would be quite difficult to change a DREB1/CBF protein from *trans*-active to *trans*-inactive. Even if we could obtain the inactive mutants by deletions, the results might be not that convincing because deletions might destroy the integrity of the protein structure and lead to some artificial phenomena. Therefore, it will be much better to start in the reverse direction, which is to transfer a *trans*-inactive DREB1/CBF to a *trans*-active one. Fortunately, we have previously reported two groups of highly homologous BnDREBs that bind to DRE, the *trans*-active BnDREBI, the *trans*-active BnDREBI and the *trans*-inactive BnDREBII [23]. And the additional two fragments in BnDREBI were not essential for the activity (Fig. 2). Then the information must be located in the homologous regions between these two groups of BnDREBs, which makes it possible to figure out the amino acids crucial for the activities of BnDREBs.

Previously, the essential region of the activity of DREB1/CBF was believed to be located in its C-terminal region [13,22]. Here, we found that the C-terminal region did play a more important role in the activity of BnDREBI, but the N-terminal, including the N and AP2 regions, also contributed to the activity (Fig. 2C). As was known, N-region of DREBs contained the nuclear localization signal [7]. It is the first time that we reported N region was involved in the activity of BnDREBI, and that Asn2 was the key residue, which might be common in DREB1/CBF.

Interestingly, the ERF/AP2 domain of BnDREBI also contributed to the activity, although its principle role is supposed to bind the DRE element. The solutions structure of the ERF/AP2 domain indicated that the β-sheets participated in binding the *cis*-element [16], but the role of the α-helix has not been identified. Here, Lys80, the key amino acid in the ERF/AP2 domain, was located in the α-helix. Therefore the possible role of this α-helix might be involved in the activity, although it seemed to be specific to BnDREBI.

In the present work, we also found that the *trans*-active regions of BnDREBI functioned in redundancy, consistent with the previous report on CBF1 [22]. First, the N-terminal (N and AP2) and the C-terminal of BnDREBI-5 functioned in redundancy. The sum of the activities of IINIC (93.9%) and INIIC (69.7%) was much higher than that of BnDREBI-5 (Fig. 2C). Second, in the C-terminal of BnDREBI-5, C1, C3 and C5 functioned in redundancy. Third, in the regions like C3 and C5 that contained more than one *trans*-active motif or amino acid, different *trans*-active motifs or amino acids also functioned in redundancy (Fig. 4).

However, things are much different, when it comes to the N-terminal and the small motifs. For one thing, N and AP2 re-

gions functioned in a linear addition pattern toward the activity of the N-terminal of BnDREBI-5, because the activity of INIIC (69.7%) was similar to the sum of II-1SN (35.8%) and II-1SAP2 (25.3%) (Fig. 2C). For another, the two Arg residues in the REEQR motif also functioned in a linear addition pattern (Fig. 4). However, in the Lys138Ser139 motif of BnDREBI, it was a little complicated. Lys138, unlike Ser139, did not show any trans-active activity, but it contributed to the activity of the motif because the double mutant, II-1KS, showed a *trans*-activation activity higher than II-1N140S (Fig. 4).

Four hydrophobic motifs have been identified to function positively towards the activity of CBF1 [22]. However, BnDREBII also contained these hydrophobic motifs (Fig. 1), but this group of proteins was *trans*-inactive. Therefore, the existence of these motifs might not be sufficient for the activity, at least in BnDREBII. Usually, hydrophobic residues or motifs are buried in the protein molecules, instead of being exposed to the surface, to maintain the structural stability of the proteins. Interestingly, the REEQR motif, Trp211, Asp221 and Asp231 identified here were adjacent to the previous reported HC2, HC3, HC4 and HC5 [22], respectively (Fig. 1). Mutations or deletions in the hydrophobic motifs might affect the right positioning of these adjacent *trans*-active amino acids, and thus led to the decrease in the activity to activate transcription.

In contrast, the polar amino acids might play more important roles in the activity of BnDREBI. Most of the key amino acids identified here are polar amino acids, except Phe148 in AtDREB1A and Trp211 in BnDREBI. The polar amino acids are usually located at the surface of the proteins, and they are widely involved in protein–protein interactions [29]. Therefore, these amino acids might participate in the interaction of DREBs with the other components needed for transcription. A previous study had shown that the possible mechanism of CBF1 to activate transcription was to recruit HAT-containing adaptor complexes to the promoter [30], and CBF1 has been demonstrated to interact with *Arabidopsis* Gcn5 and ADA2 proteins [30]. Therefore, these amino acids of BnDREBI, especially the polar amino acids, might be crucial for recruiting proteins essential for transcription, most likely the HAT-containing adaptor complexes.

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