Molecular Cloning, Phylogenetic Analysis, Expressional Profiling and *In Vitro* Studies of TINY2 from *Arabidopsis thaliana*

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A cDNA that was rapidly induced upon abscisic acid, cold, drought, mechanical wounding and to a lesser extent, by high salinity treatment, was isolated from Arabidopsis seedlings. It was classified as DREB subfamily member based on multiple sequence alignment and phylogenetic characterization. Since it encoded a protein with a typical ERF/AP2 DNA-binding domain and was closely related to the TINY gene, we named it TINY2. Gel retardation assay revealed that TINY2 was able to form a specific complex with the previously characterized DRE element while showed only residual affinity to the GCC box. When fused to the GAL4 DNA-binding domain, either full-length or its C-terminus functioned effectively as a trans-activator in the yeast one-hybrid assay while its N-terminus was completely inactive. Our data indicate that TINY2 could be a new member of the AP2/EREBP transcription factor family involved in activation of down-stream genes in response to environmental stress.

Keywords: ABA, DREB, One-hybrid, Stress, Transcription factor

Introduction

Cold and drought are adverse environmental conditions that affect the growth of plants and the productivity of crops. Genetic analysis revealed that many genes were induced to express when plants encounter such environmental stress. The responses of plants to cold and drought conditions were often similar at the molecular level for genes such as early responsive to dehydration (ERD) (Kiyosue *et al.*, 1994), cold-

regulated (COR) (Hajela *et al.*, 1990), low-temperature-induced (LTI) (Nordin *et al.*, 1993) and cold-inducible (KIN) (Kurkela and Borg-Franck, 1992) were induced by both cold and drought treatments. Two relatively independent signaling pathways responsive to cold and drought stress were identified in the literature (Xiong *et al.*, 2002; Shinozaki *et al.*, 2003). In the ABA-dependent pathway, ABA (abscisic acid) was produced upon drought and cold stress that, in turn, induced expression of various subsets of down-stream genes (Uno *et al.*, 2000; Yoshida *et al.*, 2002). In contrast, the ABA-independent pathway was characterized by a transcription regulatory system based on environmental responsive DRE-(dehydration-responsive element) or CRT- (C-repeat cis-acting element) binding trans-acting factors (Yamaguchi-Shinozaki and Shinozaki, 1994; Baker *et al.*, 1994; Wang *et al.*, 1995; Stockinger *et al.*, 1997). Genes such as *rd29A* and *kin2* were involved in both ABA-dependent and ABA-independent pathways since they possessed two types of regulatory elements required for transcription activation by different stimuli (Kurkela and Borg-Franck, 1992; Yamaguchi-Shinozaki and Shinozaki, 1993).

DRE element, TACCGACAT, first identified in the promoter region of *rd29A*, is a cis-acting DNA motif essential for cold- and drought-responsive expression of many genes including *kin1*, *cor6.6*, *rd17* as well as *rd29A* (Yamaguchi-Shinozaki and Shinozaki, 1994; Wang *et al.*, 1995; Iwasaki *et al.*, 1997). The core sequence of CRT element, CCGAC, is identical to that of DRE and is found in the promoter regions of several cold induced genes such as *cor15a*, *rd17* and *erd10* (Baker *et al.*, 1994; Kiyosue *et al.*, 1994; Iwasaki *et al.*, 1997). Two amino acid residues, valine and glutamic acid, conserved in AP2 domains of DREB subfamily members were crucial for their DNA-binding specificity (Sakuma *et al.*, 2002). Several reported stress inducible genes including CBF1, DREB2A, CBF3, and CBF4 were among members of the DREB subfamily (Stockinger *et al.*, 1997; Liu *et al.*, 1998; Gilmour *et al.*, 1998; Haarke *et al.*, 2002). CBF1 was induced

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by low temperature and over-expression of CBF1 in plants was found to activate downstream cold-responsive genes and improved freezing tolerance (Jaglo-Ottosen *et al.*, 1998). TINY was obtained several years ago through a transposon-tagging system and this semidominant mutation was found to result in significant reductions in plant heights, hypocotyl lengths and fertilities. The mutation was related to increased, or ectopic, expression of TINY (Wilson *et al.*, 1996).

Here we isolated a cDNA encoding for a TINY-like protein that was able to bind specifically to DRE element in electrophoretic mobility shift assay and showed transcription activation in yeast one-hybrid system. It was induced rapidly after ABA treatment, and also by cold and drought stress. We named it TINY2 since the gene was found to encode a protein that contained a typical ERF/AP2 DNA-binding domain, and showed close identity with TINY in the conserved domain. We suggest that TINY2 may play a role in signal transduction for cold and drought adaptation in Arabidopsis.

Materials and methods

Plant materials and treatments *Arabidopsis thaliana* (Columbia ecotype) plants were grown in fully automated growth chambers (Conviron, Winnipeg, Canada) under 16 h illumination. Plants were maintained at 22°C during the light period and 20°C during the dark period. 6-8 rosette-leaf-stage Arabidopsis seedlings were used for following treatment. ABA, 100 μM ABA was sprayed on the leaves for 8 h; NaCl, whole pots were wetted by 300 mM NaCl and kept for 8 h; heat-shock (heat), plants were pre-warmed at 37°C for 2 h before being transferred to 45°C for another 2 h; dark, plants were put into a dark chamber for 24 h; UV, plants were radiated with ultraviolet light (100 J · m^{-2}) for 6 h; dry, entire plants were uprooted, placed on filter papers and allowed to dry for 6 h; SA, 4 mM salicylic acid was sprayed on the leaves for 8 h; ethylene (eth), plants were placed in a closed chamber containing 100 ppm C$_2$H$_4$ for 24 h; cold, plants were placed in a 4°C cold room for 8 h; wound, rosette leaves were cut into ~5 mm strips and were left in the growth chamber for 8 h before being harvested for RNA isolation; control, untreated plants.

Rapid amplification of cDNA ends (RACE) and cloning of putative full-length TINY2 RACE primers were designed according to the ORF sequence predicted from NCBI (http://www. ncbi.nlm.nih.gov/) as following: RP1, 5'-AATGAAACGGGTCGA GGGAATGAGT-3'; RP2, 5'-ACCCATTTTCCCCAGTTCCTCAT CC-3'; FP1, 5'-TCTTGTGTTGGTGATGGACCTCTCG-3' and FP2, 5'-TCGGCTAACCTTGGGAACGAGTTTG3'. Experiments were carried out following the manual of GENERACER kit (Invitrogen, Sandiego, USA). PCR was performed in 50 μl reaction mix using Ex-Taq DNA polymerase. Amplification conditions were: 94°C, 30 s; 65°C, 30 s; 72°C, 1.5 min for 35 cycles with a final 10 min extension at 72°C after the last cycle. PCR products were separated on a 1.2% Agarose gel, recovered by using Gel extraction kit (QIAGEN, Chatsworth, Germany) and cloned into pGEM-T Easy vector (Promega, Madison, USA) for sequence analysis.

Phylogenetic analysis Arabidopsis genome database (NCBI, http:/ /www.ncbi.nlm.nih.gov/Blast/) was searched for all AP2/EREBP family members. Multiple sequence alignment was performed by CLUSTAL W 1.8.3 (Thompson *et al.*, 1994) and by hmmalign in HMMER package 2.3.1 (Eddy, 1998) installed locally with manual refinements and phylogenetic tree was constructed by using the Maximum-Likelihood methods with bootstrapping repeated 100 times.

RT- and QRT-PCR analysis Total RNA was isolated from pooled *Arabidopsis* plant samples using the RNeasy plant mini kit (QIAGEN, Madison, Germany) and was reverse transcribed using SuperScript^{TM} First-Strand Synthesis System for RT-PCR (Invitrogen, Sandiego, USA). Total RNA extracted from *Arabidopsis* seedlings (6-8 rosette leaf-stage) that received various environmental treatments were quantified at 260 nm with a spectrophotometer and 3 μg of the RNA was reverse transcribed in a total volume of 20 μl. Primers for *UBQ10* amplification were added as the internal control together with gene-specific primers. Amplified cDNA fragments were analyzed on 1.2% Agarose gel with the Gel Doc 1000 software (Bio-Rad, Richmond, USA). QRT-PCR was carried out using the SYBR green PCR kit (Applied Biosystems, Foster City, USA) in a DNA Engine Opticon-Continuous Fluorescence Detection System (MJ Research, Watertown, USA). Samples were analyzed in triplicates with independent RNA samples and were quantified by the comparative cycle threshold method.

Gel shift assay The coding region of TINY2 was cloned into the pGEX-4T-1 vector and expressed in *E. coli* BL21 codon-plus cells (Amersham Pharmacia Biotech, Uppsala, USA). GST fusion protein was purified with Glutathione Sepharose 4B (Amersham Pharmacia Biotech). Oligonucleotides containing either the authentic or series of mutated DRE/GCC motifs were synthesized by Sunbiotech (Shanghai, China). Gel shift assay was performed in a total volume of 20 μl, in which there were 100 ng GST-fusion protein, 2 pmol of ^{32}P end-labeled oligonucleotides, 1 μg poly (dA-dT), and DNA binding buffer (25 mM HEPES-KOH, pH 7.5, 10% Glycerol, 4 mM NaCl, 1 mM DTT, 40 mM KCl, 0.5 mM EDTA). After incubated for 2 h at room temperature, the reaction mixture was run on 6% non-denaturing PAGE in 0.5XTBE (45 mM Tris-borate, 1 mM EDTA). Binding activities were quantified using the Typhoon 9200 PhosphorImager (Amersham Pharmacia Biotech).

Yeast strain, yeast/*E. coli* shuttle vector and one-hybrid assay Yeast (*Saccharomyces cerevisiae*) strain EGY48 (MATα, his3 trpl ura3-52 leu::pleu2-lexAop6) and yeast/*E. coli* shuttle vector pG222 and pYF503 were given to us by Professor Hongwei Xue from the Chinese Academy of Sciences. The recently published yeast one-hybrid system (Ye *et al.*, 2004) was employed to identify the trans-activation ability of TINY2. Primers with *EcoR* or *Bgl* restriction sites were designed to generate full-length or partial sequences of TINY2, respectively using the cDNA as the template. All cloned fragments were sequence and the right clones were then digested with respective endonucleases before ligating into predigested vector pYF503. The recombinant vector was then transformed into yeast strain EGY48 harboring a reporter vector pG222. Clones growing on selective media were picked out for blue/white color selection as described (Ye *et al.*, 2004).
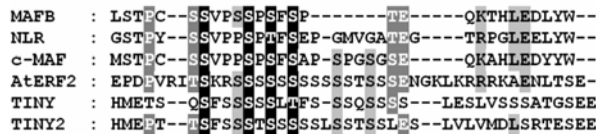
(A)

(B)
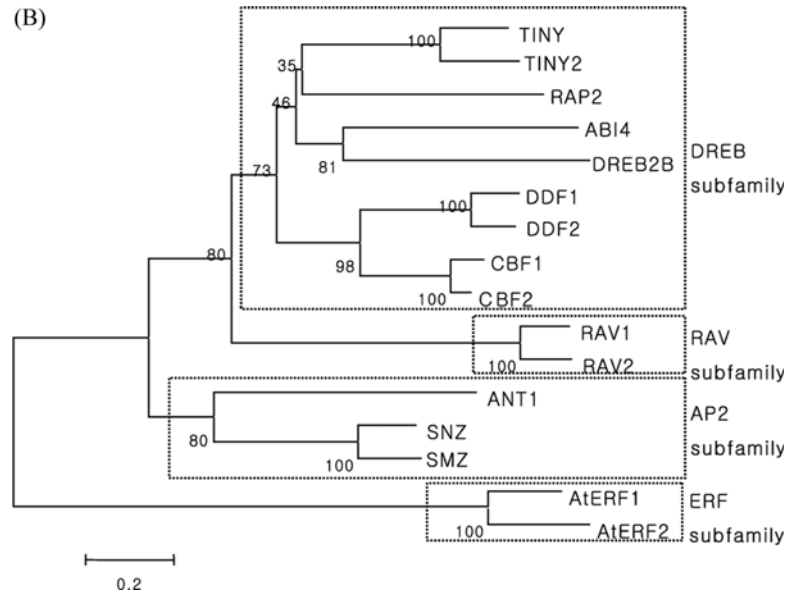
(C)

Fig. 1. Sequence comparison and phylogenetic analysis of TINY2. A, Multiple sequence alignment of TINY2 and several reported DREB subfamily transcription factors in Arabidopsis. B, Phylogenetic tree constructed using TINY2 together with eight closely related genes within the same subfamily as well as a few representative genes from other subfamilies. The tree was constructed by using amino acid sequence of the conserved AP2 domain with the Maximum-Likely hood method. Bootstrap values shown at the branches indicate the significance level. C, TINY2 has a typical serine- and threonine-rich transactivation motif in its C-terminus that is homologous to TINY, AtERF2, NRL, cMAF and MAFB.

Results and Discussion

Cloning and phylogenetic analysis of TINY2 The Arabidopsis ATH1 microarrays were first probed with RNA samples prepared from Arabidopsis seedlings after various biotic or abiotic stresses and a cDNA that was expressed more than ten-fold higher upon ABA treatment was recovered (data not shown). After cDNA RACE, we obtained a putative full-length gene of 1215 bp that encoded a polypeptide of 236 amino acid residues. It was submitted to GenBank under the accession number of AY940160. Multiple sequence alignment showed that TINY2 shared a conserved AP2 domain with several AP2 family transcription factors in Arabidopsis that were previously experimentally characterized (Fig. 1A). Phylogenetic analysis showed clearly that it belongs to the DREB subfamily with high sequence identity to TINY and to RAP2, ABI4 and DREB2B (Fig. 1B) (Okamuro et al., 1997; Finkelstein et al., 1998). Sakuma and colleagues divided the Arabidopsis AP2/ERF transcription factor family into five subfamilies including DREB, ERF, AP2, RAV and others. They put the sequence corresponding to TINY2 in DREB subfamily together with TINY and other related genes (Sakuma et al., 2002). Further sequence analysis showed that TINY2 contained a typical serine- and threonine-rich motif at the C-terminus similarly to that of TINY, AtERF2 and also to NRL, cMAF as well as MAFB (Fig. 1C) (Blank and Andrews, 1997; Fujimoto et al., 2000; Friedman et al., 2004).

Expression profiling of TINY2 RT-PCR analysis showed that TINY2 was expressed in very low levels in untreated Arabidopsis plants. However, it was significantly induced after ABA, cold, wound, NaCl and drought treatment (Fig. 2A). The expression patterns of TINY2 were further quantified by QRT-PCR analysis as in Fig. 2B. It was amplified in 26.18 ± 0.27, 27.28 ± 0.06 and 27.78 ± 0.35 cycles, respectively after ABA, cold and wounding treatment that corresponded to about 12-, 5- and 4-fold increases when compared to that of untreated control plants (Fig. 2B and Supplemental 1). Results obtained from RT- and QRT-PCR analysis agreed well with each other and also with that of gene chip study (data not shown).
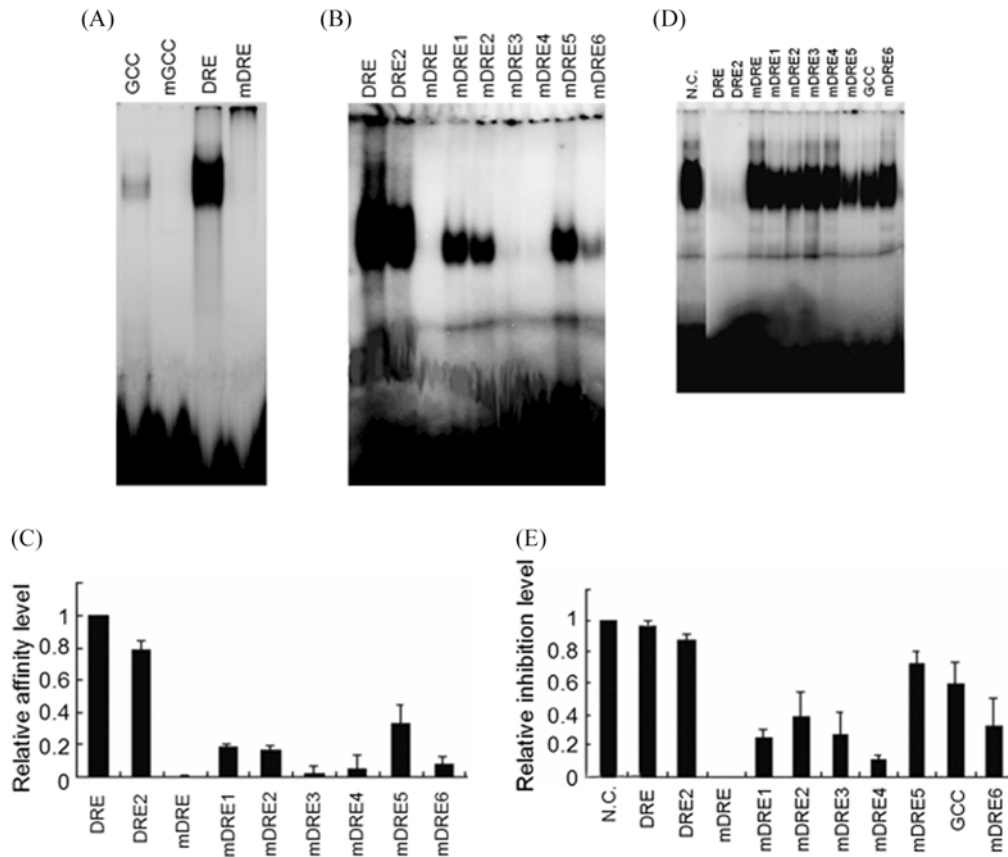
TINY2 bind specifically to authentic DRE motif, but not to GCC or mDREs When TINY2 was expressed in E. coli and used in gel mobility shift assay, we found that the protein displayed high affinities to the sequence ACCGAC (DRE) with about 15% affinity to GCCGCC (GCC) box. TINY2 lost completely its affinity to DRE box when the G and Cs present in the last four bases of the core sequence were changed to Ts and lost completely its affinity to mGCC with two T/G substitutions (Fig. 3A). Further analysis showed that over 95% of the binding activities were lost when the third C was changed to T or the fourth G to T as in mDRE3 and mDRE4, respectively (Fig. 3B and C). Competitor inhibition analysis produced similar results. When non-radial labeled DRE or
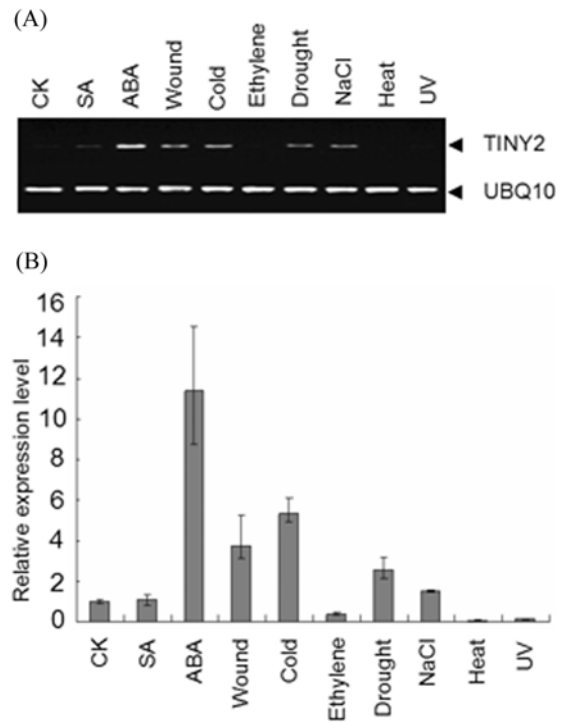
Fig. 2. TINY2 is induced differentially by various biotic or abiotic stimuli. Shown in A and B were RT- and QRT-PCR results obtained by using RNA templates that were prepared from Arabidopsis seedlings after different treatments, respectively (SA, salicylic acid; ABA, abscisic acid; UV, ultraviolet). Primers for TINY2 and UBQ10 were included in the same PCR reaction, and amplified products were separated on 1.2% agarose gel. Note that the UBQ10 gene was already saturated at this point while the signal for TINY2 was still very weak even after ABA, wound and cold treatments. In QRT-PCR analysis, expression of TINY2 in Arabidopsis seedlings after various treatments were compared relatively to that of untreated control plants (CK) taken as 1.

DRE2 (40 times more than the amount of radial labeled probes) were used as competitors, binding of the protein to wild-type DRE motif was almost abolished (Fig. 3D, E). However, when various mutant probes with one or more bases substituted were used as cold competitors, we found that binding of TINY2 to its cis-element was affected very differently. The sequence labeled as mDRE was totally inactive since it did not compete with the authentic DRE for protein binding (Fig. 3D, E). Sequences of mDRE4 showed 10% while that of mDRE5 showed about 80% competitive inhibition when compared to the wild-type DRE (Fig. 3D, E). Sakuma et al.(2002) reported that the 4th C, 5th G, and 7th C of DRE (TAC<u>CGA</u>CAT) were essential for sequence-specific interactions with the DREB proteins.

TINY2 is able to activate transcription in the yeast one-hybrid assay When the open reading frame (ORF) of TINY2 was fused to GAL4 DNA-binding domain and was further transferred into yeast cells carrying the LacZ reporter

Fig. 3. DNA-binding affinity studies using TINY2 and related sequence motifs. A, TINY2 binds specifically to DRE, very weakly to GCC and is completely inactive to mGCC and mDRE motifs. B, Analysis of binding affinities of TINY2 to the conserved DRE, DRE2 and to a series of one-base substituted sequences. C, Relative DNA-binding affinities as quantified by using the PhosphorImager. Shown are the mean ± SE of three independent gel-shift experiments. D, Only DRE, DRE2 and to some extent mDRE5 as well as GCC are able to compete with radial labeled DRE sequence. The line marked as N.C. comes from another independent experiment and means that no competitor was mixed with the radial labeled DRE probes. E, Quantitative analyses of the competitiveness of different competitors by using the PhosphorImager. A total of 50 ng of *in vitro* expressed and affinity-column purified TINY2 proteins were used for all binding reactions. 2 pmol of ^{32}P end-labeled oligonucleotides were used as the probe in each reaction. Forty-time excess amounts of unlabeled oligonucleotides were mixed with 2 pmol of ^{32}P end-labeled probes in competition studies. Sequences of different probes: DRE, 5'-ATACTACCGACAT-3'; DRE2, 5'-ATACTGCCGACAT-3'; mDRE, 5'-ATACTACTTATAT-3'; mDRE1, 5'-ATACTTCCGACAT-3'; mDRE2, 5'-ATACTATCGACAT-3'; mDRE3, 5'-ATACTACTGACAT-3'; mDRE4, 5'-ATACTACCTACAT-3'; mDRE5, 5'-ATACTACCG TCAT-3'; mDRE6, 5'-ATACTACCGATAT-3'; GCC, 5'-TAAGAGCCGCCACT-3'; mGCC, 5'-TAAGATCCTCCACT-3'.

construct, the recombinant protein activated *LacZ* transcription as identified by the blue/white colony selection method (Fig. 4). Two more constructs were made with either the N-terminus (include amino acid residues from 1-135 that contains the whole AP2 domain) or the C-terminus (include amino acid residues from 136-236, immediately downstream of the AP2 domain) of TINY2 fused to GAL4 DNA-binding domains and their abilities in transcription activations were tested by using the same *in vitro* assay system. The putative transactivation domain that showed significant sequence homology with a number of known transcription activators such as AtERF2, NRL, cMAF and MAFB, was able to activate *LacZ* transcription (Fig. 4). By contrast, the region upstream of AP2 domain was completely inactive in the assay

(Fig. 4). Previously it was reported that amino acid sequences rich in serine and threonine residues possessed abilities of transcription activation (Gerber *et al.*, 1994). Our results suggest that the C-terminus of TINY2 may be responsible for transactivation of target genes.

In summary, we isolated a cDNA named TINY2 from *Arabidopsis* seedlings that was induced by ABA, cold and drought treatment. TINY2 can bind specifically to DRE motif and can functionally activate transcription of a reporter gene, presumably through the function of its serine- and threonine-rich transactivation domain, in an *in vitro* assay. Characterization of TINY2 may help understand plant stress tolerance mechanisms.
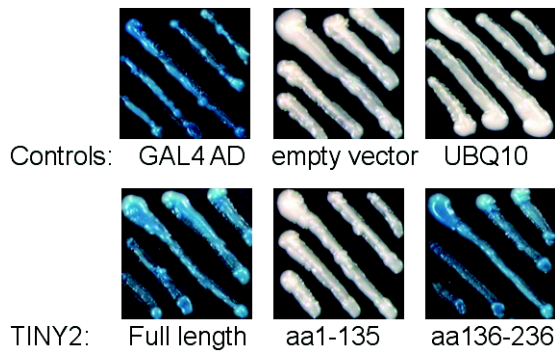
Fig. 4. Either the entire ORF of TINY2 or its C-terminus provided the trans-activation domain needed for reporter gene activation in the yeast one-hybrid assay. Plasmid DNA from various constructs was transformed into yeast cells and the abilities of transcription activation of the recombinant protein was identified via blue/white selection. Note that no blue color is observed in yeast cells transformed with either the empty vector (contains only the GAL4 DNA-binding domain), with a full-length UBQ10 gene or the N-terminus (from amino acid 1-135) of TINY2 ligated to the GAL4 DNA-binding domain.

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