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Short Communication

Identification and properties of a small protein that interacts with a tobacco bZIP-type transcription factor TBZF

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Abstract The tobacco bZIP transcription factor, TBZF, functions in leaf senescence and floral development. In order to identify TBZF-interaction partner(s), we screened a cDNA library constructed from leaves of tobacco (*Nicotiana tabacum* cv. Xanthi) by the yeast two hybrid method. Among 16 clones initially identified, one clone was further characterized because of its highly specific binding to the bait. The full length cDNA of 776 bp encoded a polypeptide of 12.5 kDa, which was designated NtTIP1 (*Nicotiana tabacum* TBZF-Interaction Protein 1). A high similarity was scored to potato CI21A, a member of the ASR/CI21 family of proteins, most of which are induced by biotic and abiotic stresses. NtTIP1 was revealed to be localized in the nucleus by fluorescence assay of NtTIP1-GFP fusion protein in onion epidermal cells. Both *NtTIP1* and *TBZF* transcripts accumulated at high levels in flowers and senescing leaves. The expressional correlation was also observed particularly after 6 h cold stress, and after ABA, JA and ethylene treatments. The results suggest that NtTIP1 and TBZF form a complex *in vivo*, and they possibly function in flower development and stress response.

Key words: bZIP, ASR/CI21 family, flower development, *Nicotiana tabacum*, abiotic stress

The bZIP transcription factors form a large diverse family. More than 200 genes for bZIPs have so far been identified in plants and the number may further increase in future. Even in a single species, the genes constitute a superfamily. For example, *Arabidopsis* genome analysis revealed that this organism contains almost 75 forms (Riechmann and Ratcliffe 2000). The bZIPs are characterized by a basic region, that establishes sequence-specific DNA binding, and an adjacent section of heptad leucine repeats that form two parallel α helices arranged as a coiled-coil. This enables dimerization of two proteins, which determines the orientation of the basic domains within the major groove of the DNA (Landschulz et al. 1988; Vinson et al. 1993). The bZIPs have been suggested to form homodimers and heterodimers and also to interact with non-bZIP type proteins to regulate transcription. Identification of proteins interacting with bZIP proteins should thus provide valuable clues to determine regulatory mechanisms underlying bZIP dependent gene regulation. For example, yeast two hybrid screening identified seven genes encoding 14-3-3 proteins, which specifically interact with RSG, a bZIP-type transcription factor involved in the gibberellin signaling pathway (Igarashi et al. 2001). Such an interaction between bZIPs and 14-3-3 proteins appears to be involved in translocation to the nucleus (Igarashi et al. 2001).

Functional roles of bZIP transcription factors have been documented in response to light (Oyama et al. 1997), hormones (Fukazawa et al. 2000), and biotic (Zhou et al. 2000) as well as abiotic stresses (Ito et al. 1999). They are also involved in cell proliferation (Mikami et al. 1995) and developmental processes (Chuang et al. 1999). In our previous report, we described identification of two tobacco bZIPs, TBZF and TBZ17 (Yang et al. 2001). Their encoding genes were originally isolated from aged tobacco leaves and found to show 73% identity with each other. Both proteins are located in nuclei where they preferentially bind to DNA fragments through the G-box motif and show transactivation activity. *In situ* hybridization analysis revealed transcripts of *TBZF* and *TBZ17* to be predominantly located in guard cells and vascular tissues of senescing leaves. Accumulation of *TBZF* transcripts could also be shown in developing flower organs. These results suggest that TBZF and TBZ17 are both

Abbreviations: bZIP, basic region leucine zipper protein; 3-AT, 3-aminotriazole; SD, synthetic dextrose; ASR, ABA/water stress/ripening-induced; CI21, Cold-induced 21.

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involved in controlling transcription of genes related to functions of guard cells in senescing leaves and that TBZF bifunctionally acts in floral development (Yang et al. 2001).

The yeast two-hybrid screening was performed using the HybriZAP vectors Cloning System (Stratagene). In order to obtain a prey population, an expression cDNA library was generated in pAD-GAL4 (Stratagene) using mRNA isolated from tobacco leaves, so that fusion products of the GAL4 activation domain and proteins from tobacco cDNAs could be produced. The original library contained approximately 2×10^7 independent clones. Plasmids were obtained by *in vivo* excision using a helper phage (ExAssist, Stratagene) and employed for subsequent transformation of yeast. For the bait construct, the entire coding region of *TBZF* was cloned into the pBD-Gal4 Cam vector to produce a fusion of the GAL4 DNA binding domain and TBZF (pBD-TBZF). Yeast strain Y190 was first transformed with pBD-TBZF and it was confirmed that pBD-TBZF did not activate reporter genes. The library plasmids described above were then introduced into yeast harboring pBD-TBZF by the polyethylene glycol/lithium acetate method. Transformants (1×10^6) was harboring the bait and prey plasmids were screened on plates containing 20 mM 3-aminotriazole (3-AT) in synthetic dextrose (SD) medium without Trp, Leu and His. Interaction assays were performed on plates containing 0, 25, 50 or 75 mM 3-AT in SD medium without Trp, Leu and His. For qualitative β-galactosidase assessment, activity was detected in filter-lifting assays using 5-bromo-4-chloro-3-indolyl β-D-galactoside. For quantitation, yeast cells were grown overnight in SD selection medium, diluted 1 to 5 in YPDA medium and grown for an additional 3 to 5 h at 30°C. Three independent colonies were assayed for each construct. As the substrate *o*-nitrophenyl β-D-galactopyranoside was applied and β-galactosidase activity (U) was calculated as $U=1{,}000\times(OD_{420})/[\text{Time (min)}\times\text{Vol (ml)}\times(OD_{600})]$.

In order to identify proteins interacting with TBZF, a cDNA library containing 10^7 independent clones was screened using the yeast two-hybrid system. Prior to screening, pBD-TBZF fusion protein was confirmed not to activate transcription of reporter genes by itself (data not shown). A total of 1.8×10^6 transformants were screened for their ability to grow on medium without His and in the presence of 20 mM 3-AT. This initial screening identified 135 colonies, which were subsequently tested in the qualitative β-galactosidase assay. Sixty three colonies were positive for activation of both *HIS3* and *LacZ*. Plasmid DNAs were prepared from these colonies and transformed into the Y190 strain to confirm whether the activation was indeed due to the presence of the fusion protein. The results showed 16 clones to be able to activate *HIS3* and *LacZ* in the presence of pBD-TBZF

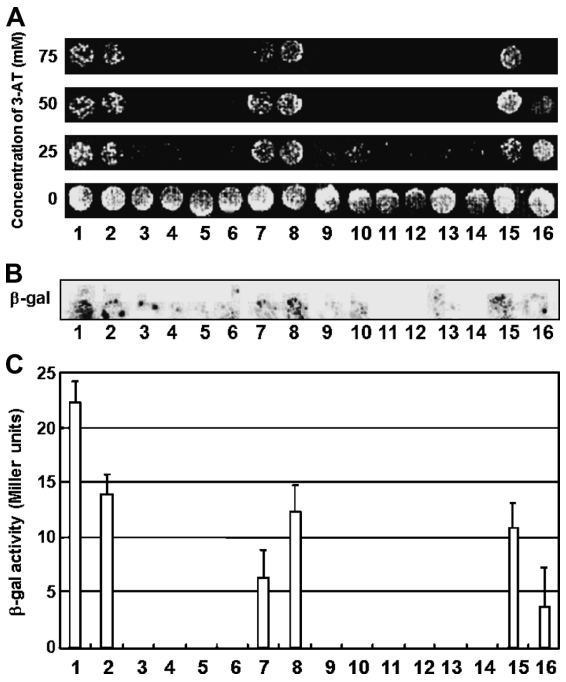

Figure 1. Clones encoding the TBZF-interaction proteins. (A) Yeast two-hybrid screening of tobacco leaf cDNA library for the identification of the clones encoding TBZF interacting proteins. The yeast cells carrying pBD-TBZF were transformed with the cDNA library. The colonies grown on SD medium (-Leu/Trp/His) containing 20 mM 3-AT were selected. The clones carrying pAD-TBZ17 and pAD-TBZF were eliminated and the residual candidate clones were retransformed to yeast cells carrying pBD-TBZF. The resulting 16 clones were tested for their strength of TBZF-interaction activity. BLAST results of cDNA clones 1–16 are listed in supplementary Table 1, clone 1 corresponds to NtTIP. (B) β-galactosidase assay of the clones. Colonies grown on SD medium (-LTH, 0 mM 3-AT) were used for filter-lift β-galactosidase assay. (C) Quantitative β-galactosidase activity assay. Three individual transformants were used to measure β-galactosidase activity. Vertical bars represent standard deviations.

(Figure 1A, B, Supplementary Table 1). Of them, clones 1, 2, 7, 8, 15 and 16 strongly activated the reporter genes. In this study, clone 1, showing the strongest interaction with TBZF (Figure 1C), was subjected to further analysis. Vital growth of the transformant carrying pBD-TBZF and clone 1 was observed even in the presence of 3-AT at a concentration as high as 75 mM (Figure 1A). First, a full length cDNA was isolated, since the clone selected by the two-hybrid screening was partial, using primers for the vector and the cDNA region. RACE-PCR yielded a cDNA in size of 776 bp which contained the entire ORF. This cDNA, designated *NtTIP1* (*Nicotiana tabacum* TBZ Interacting Protein 1, accession number HE664126) encoded a putative protein consisting of 110 amino acids with a predicted molecular mass of 12.5 kDa, which showed a high identity to tomato and potato proteins (Figure 2A). A phylogenic tree indicated NtTIP1 to be closely related to potato CI21A, a member of the ASR/CI21 family (Figure 2B). During the two-hybrid screening, we identified TBZF and TBZ17 as interacting

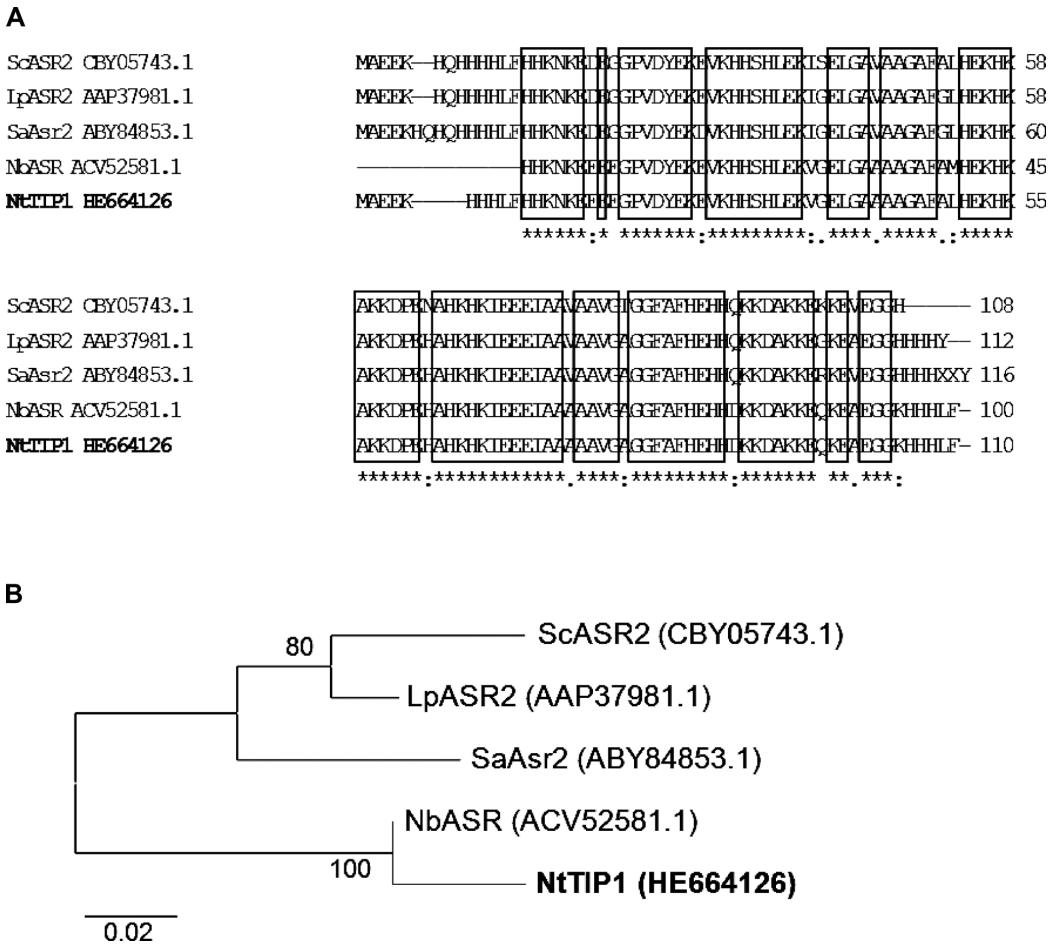

Figure 2. NtTIP1 is a member of CI21/ASR family. (A) Amino acid sequence alignment of NtTIP1 and other CI21/ASR family proteins. (B) A phylogenic tree among CI21/ASR family proteins. Amino acid sequences are from SaASR2 (*Solanum arcanum*), ScASR2 (*Solanum chilense*), LpASR2 (*Lycopersicon peruvianum*), NbASR (*Nicotiana benthamiana*), and NtTIP1.

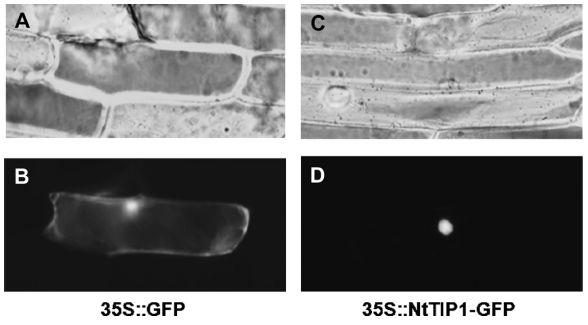

Figure 3. Nuclear localization of NtTIP1 in onion epidermal cells. Onion bulbs were bombarded with gold particles coated with pGFP2 (A, B) and pNtTIP1::GFP (C, D) plasmids, respectively. Differential interference contrast images (A and C) and fluorescence images (B and D).

proteins (data not shown). The above result clearly indicates that a non-bZIP protein, NtTIP1, efficiently interacts with TBZF, and that TBZF forms hetero- as well as homo- dimer.

To reveal the subcellular localization of NtTIP1, the entire coding region of *NtTIP1* was subcloned into pGFP2 (provided by Drs. Chua and Spielhofer) at *Xba*I and *Kpn*I sites, yielding pNtTIP1::GFP. Onion bulbs cut into 9 cm^2 were bombarded with gold particles (Bio-Rad) coated with pGFP2 or pNtTIP1::GFP (Hara et al. 2000). After 6 h incubation at room temperature under complete darkness, the epidermal cell layers were viewed using a microscope (Olympus Provis AX70) equipped with a fluorescence module. Transformation with the plasmid expressing GFP alone showed fluorescence throughout the cell (Figures 3A, B), whereas the green fluorescent signal was detected exclusively in the nucleus in cells transformed with the plasmid expressing the NtTIP1-GFP fusion protein (Figures 3C, D).

Tomato *ASR1* transcripts are reported to be induced by water deficit and during fruit ripening (Iusem et al. 1993; Rossi and Iusem 1994). Other family members, potato *ci21A* and *ci21B*, have been shown to be induced upon cold-, ABA- and osmotic-stresses (Schneider et al. 1997; van Berkel et al. 1994). Expression of the *ASR/CI21* gene family is also reported to be regulated during developmental processes (Gillapsy et al. 1993; Iusem et al. 1993; Meekswager 1993; Thomas 1993).

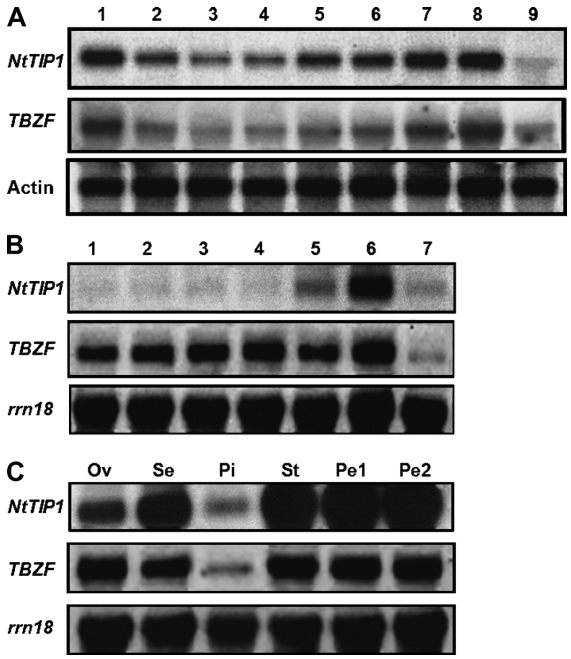

Figure 4. Tissue-specific expression of *NtTIP1* and *TBZF*. (A) Expression profiles of *NtTIP1* and *TBZF* in flowering stage tobacco plants. Total RNA was isolated according to the aurin tricarboxylic acid (ATA) method (Nagy et al. 1988), or by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method (Chomczynski and Sacchi 1987). Total RNAs (20 μg per each lane) were separated by electrophoresis in formaldehyde–1.2% agarose gels, blotted onto Hybond N membranes (Amersham) and subjected to crosslinking by UV irradiation. Hybridization was carried out as described previously (Yang et al. 2001), using respective probes, ORF for *NtTIP1* and 3′-UTR for *TBZF*. Lane 1, flower; lanes 2 to 8, indicate leaves of different developmental stages. Leaf positions from the tip to the base of flowering stage tobacco plants are serially numbered with leaves 6 to 8 being senescent; lane 9, root. Tobacco *actin* cDNA was used as a loading control. (B) Expression of *NtTIP1* and *TBZF* along with flower development. Total RNAs were extracted from flowers at various developmental stages. Flower stages were serially numbered from young bud (stage 1), bud (stage 2), mature bud (stage 3), young flower with closed petals (stage 4), young flower with open petals (stage 5), mature flower with completely open petals (stage 6) and faded flower (stage 7). *rrn18*-cDNA was used as a loading control. (C) Tissue specificity of *NtTIP1* and *TBZF* in tobacco flower organs. Ovule (O), sepal (Se), pistil (Pi), pink part of petal (Pe1) and lower white part of petal (Pe2) were prepared from flowers at stage 6. *rrn18*-cDNA was used as a loading control.

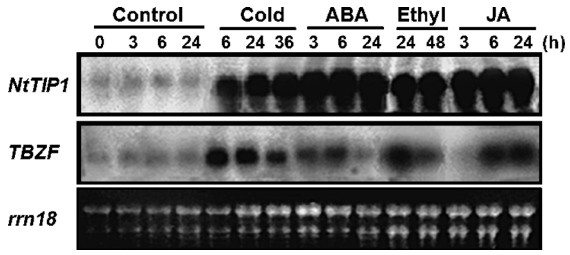

Figure 5. Transcript accumulation of *NtTIP1* and *TBZF* in tobacco plants subjected to cold stress and to hormonal treatments. Tobacco seedlings, grown in controlled environment chambers at 25°C under continuous white light (90 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR)), were transferred to liquid 1/2 strength MS medium and maintained for another 2 days before treatment. Then the seedlings were exposed to 4°C (Cold), or treated with 20 μM abscisic acid (ABA), 20 μM ethylene (Ethylene) or 20 μM jasmonic acid (JA). For ethylene treatment, plants were exposed to 10 μl/l ethylene gas in special glass containers. At each time point, 2nd and 3rd leaves from the tops of plants were harvested, frozen in liquid nitrogen and stored at −80°C until use.

Therefore, the expression of *NtTIP1* was analyzed by northern hybridization along with that of *TBZF*. *NtTIP1* expression was highly correlated with leaf age, as similar to that of *TBZF* (Figure 4A; Yang et al. 2001). Furthermore, *NtTIP1* and *TBZF* transcripts were highly abundant in flower organs (Figure 4A), while the timing of their transcript accumulation during flower development was different (Figure 4B). Namely, *NtTIP1* expression started specifically in young flower and mature flower of stage 5 and 6, whereas *TBZF* was expressed throughout the flowering stages except in faded flower of stage 7 (Figure 4B). Moreover, in mature flower, the abundance of *NtTIP1* and *TBZF* transcripts was very much correlated; i.e., lower in pistils and highly abundant in all other flower tissues (Figure 4C). The expression of *NtTIP1* and *TBZF* was induced by low temperature, but with different profiles (Figure 5). Their expression is also responsive to the phytohormones ABA, ethylene and JA, again with different profiles (Figure 5).

This paper documents the isolation and characterization of a gene encoding NtTIP1, non-bZIP protein, as a TBZF-interaction partner. In plants, a notable finding is RSG, a rice bZIP transcription factor that interacts with a 14-3-3 protein, regulating genes involved in the gibberellin biosynthesis pathway (Igarashi et al. 2001). To our knowledge, NtTIP1 is the second such case, and its complex with TBZF possibly functions in regulation of genes related to development and stress responses. Our findings also provide a clue to physiological functions of ASR/CI21 family proteins since their induction profile is consistent with that for NtTIP1, including ABA- and cold-induction. It is conceivable that small proteins of this family interact with various bZIPs, thereby controlling their transcriptional activity. Finally, it is worth to mention that, in addition to TBZF/NtTIP1 interaction, TBZF was confirmed to interact not only with itself to form a homodimer but also with TBZ17, a related bZIP protein as heterodimer (Yang et al. 2001, data not shown). This is consistent with a report that TBZF forms heterodimers with other bZIPs involved in regulation of flower development (Strathmann et al. 2001). Taken together, the available information indicates that TBZF can interact with several bZIPs and non-bZIP (NtTIP1) proteins in a complex manner. Although the exact interaction mode has yet to be ascertained, it is highly probable that such transcription complexes play roles in switch-on/off of relevant genes.

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