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Short Communication

A comparative analysis of basic helix-loop-helix proteins, AtPTF1 and NtWIN4, with reference to plastid localization

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Abstract Basic helix-loop-helix (bHLH) proteins constitute one of the most abundant types of transcription factor in both plants and animals. They are considered to be localized in the nucleus, but recent surveys have revealed two exceptions, NtWIN4 from tobacco and AtPTF1 from Arabidopsis, both reported to localize to plastids. Their specific subcellular localization was suggested to be the result of structural change, whose analysis might provide clues to understanding protein evolution. Consequently we performed comparative analyses on localization features of both NtWIN4 and AtPTF1 using GFP-tagged fusion proteins. While epifluorescence from NtWIN4-GFP was clearly observed in plastids, that from AtPTF1-GFP was unexpectedly seen only in nuclei. Subsequent transcription assays using a dual-luciferase system indicated that AtPTF1 possesses clear nuclear transcriptional repression activity. It is concluded that, in contrast to the previous paradigm, AtPTF1 is a nuclear transcriptional repressor, and therefore that NtWIN4 is the only non-nucleus resident bHLH protein.

Key words: *Arabidopsis thaliana*, basic helix-loop-helix protein, plastid localization, *Nicotiana tabacum*, nuclear transcriptional repressor.

In all eukaryotic phototrophs, many nuclear encoding proteins are incorporated into specialized organelle plastids (e.g. chloroplasts), and function in diverse areas of regulation, including transcription of plastid genes and biosynthesis of chlorophylls, carotenoids and fatty acids (Buchanan et al. 2000). Several DNA-binding proteins active in transcriptional regulation in chloroplasts have been identified, with suggested origins from both prokaryotic and eukaryotic ancestors. Proteins of probable eukaryotic origin from their primary structures are exemplified by PD3 from pea, CND41 from tobacco cultured cells, and PEND from several plant species (Sato 2001; Kodama 2007). PD3 binds to AT-rich sequences and is involved in nucleoid structures (Sato 2001), while CND41 possesses both non-specific DNA binding and protease activity (Nakano et al. 1997). PEND, a polypeptide containing a basic region plus a leucine-zipper region, is present in the inner envelope membrane of developing chloroplasts and specifically binds to TAAGAAGT (Sato et al. 1998; Sato and Ohta 2001). Another example is Arabidopsis PTF1 (AtPTF1), which is a nuclear-encoded basic helix-loop-helix (bHLH) protein reported to function as a transcription factor in plastids (Baba et al. 2001).

Basic helix-loop-helix proteins were first identified as E12 and E47 transcriptional factors in the mouse (Murre et al. 1989). The bHLH domain consists of approximately 60 amino acids with two different functional regions. The basic region located at the N-terminal end binds to a consensus hexanucleotide E-box (CANNTG) while the helix-loop-helix region at the C-terminal end of the domain functions to form homo- and/or hetero-dimers (Murre et al. 1989; Ferre-D'Amare et al. 1994). A number of bHLH proteins have now been identified in both plants and animals, and assigned to a group of transcriptional factors located within nuclei (Garrell and Modolell 1990; Quail 2000). However, as a rare and exceptional case, cellular localization other than in the nucleus has been reported for AtPTF1, in plastids. AtPTF1 was first isolated by yeast one-hybrid screening using a modified *psbD* light-responsive promoter (LRP and found to bind to the ACC repeat region of the LRP sequence, localized in chloroplasts) (Baba et al. 2001). There is evidence that it regulates LRP transcription in plastids, and involvement in transcription of the *psbD* LRP gene has been suggested (Baba et al. 2001). Recently, we identified another bHLH protein that is translocated into plastids and participates in hypersensitive cell death in tobacco plants (Kodama and Sano 2006). This protein, designated as NtWIN4, was proposed to be translated from the second Met, which facilitates the N-terminus as a plastid transit signal,

Abbreviations: bHLH, basic helix-loop-helix; CMV, cauliflower mosaic virus; DAPI, 4′,6-Diamidino-2-phenylindole dihydrochloride; GFP, green fluorescent protein; LRP, light-responsive promoter; R-Luciferase, *Renilla* luciferase.
This article can be found at http://www.jspcmb.jp

resulting in formation of a 17 kDa plastid-localized protein (Kodama and Sano 2006). It is not a homolog of AtPTF1, and in this report we document studies of mechanisms of plastid localization showing that AtPTF1, in fact is localized to the nucleus, where it functions as a transcription repressor.

The full-length *AtPTF1* and plastid-localized type *NtWIN4*$^{27\text{-}247}$ (Kodama and Sano 2006) were subcloned into the SalI/NcoI sites of a CaMV35S-sGFP(S65T)-nos vector, harboring a synthetic gene for improved green fluorescent protein sGFP(S65T) driven by the cauliflower mosaic virus (CMV) 35*S* promoter and an *NOS* terminator (Chiu et al. 1996). Onion epidermal cell layers were bombarded with gold particles (Bio-Rad Laboratories, Hercules, CA) coated with appropriate vector constructs, and after incubation at 28°C for 6–12 h in the dark, samples were viewed under a microscope (Olympus PROVIS AX70) equipped with a fluorescence module. For DNA staining, samples were incubated with 1 mg/ml 4′,6-diamino-2-phenylindole (DAPI) solution before observation. The GAL4BD-PTF1 effector plasmid was constructed by fusing a cDNA encoding AtPTF1 with the GAL4 DNA binding domain in a yy64 vector, a derivative of pMA560 (Yamamoto and Deng 1998). *AtPTF1* was subcloned into the BglII/SalI sites and yy64 vector alone was used as a control. The reporter plasmid, yy96, contained a luciferase gene placed under control of the GAL4 binding site (Yamamoto and Deng 1998). An internal control plasmid, containing a *Renilla* luciferase (R-Luciferase) gene placed under control of the 35*S*-CaMV promoter was used to normalize for differences in bombardment efficiency. Seven-day old MM2d cells were plated on MS agar and bombarded with plasmids (effecter : reporter : reference = 2 : 2 : 1) coated on a 1.0 μm microcarrier, under a vacuum of 28 inches of mercury using a helium pressure of 1,100 psi (PDS 1000, Bio-Rad). Cells were placed 6 cm from the stopping screen. After bombardment, they were incubated in the dark at 28°C for 22 h. Luciferase and R-luciferase activities were assayed using a dual-luciferase assay kit (Promega, Madison, WI) according to the manufacturer's instructions and chemical luminescence was measured using a luminometer (Lumat LB9507, Berthold Technologies, Bad Wildbad, Germany).

AtPTF1 transcripts were predominantly found in leaves, but also detected at much low levels in stems, flowers and roots (Baba et al. 2001), implying that AtPTF1 might be involved in photosynthesis. Indeed, GFP-fusion AtPTF1 was reported to be localized only in chloroplast of tobacco guard cells (Baba et al. 2001). Based on these observations, it was proposed that AtPTF1 proteins were translocated into plastids (Baba et al. 2001). In our previous studies, we showed that GFP-fusion NtWIN4 was clearly localized into chloroplasts of tobacco leave cells, and also into plastids of onion epidermal cells (Kodama and Sano 2006). In order to compare plastid localization patterns between AtPTF1 and NtWIN4 proteins, we visually examined cellular localization of GFP-fusion AtPTF1 and NtWIN4 proteins in parallel. Vectors with *AtPTF1-GFP* or *NtWIN4-GFP* were bombarded into onion epidermal cells, and cells were observed under light interference contrast and epifluorescence. Nuclei were identified by 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) staining. In contrast to the control GFP localization in both the nucleus and cytoplasm (Figure 1, left panel), AtPTF1-GFP was solely localized only in nucleus and not in any organelles, including plastids (Figure 1, second panel from the left). This was confirmed by merging GFP with DAPI images, showing a complete match. NtWIN4-GFP, in contrast, was clearly localized in plastids (Figure 1, second panel from right), giving a similar fluorescence pattern to the plastid-resident

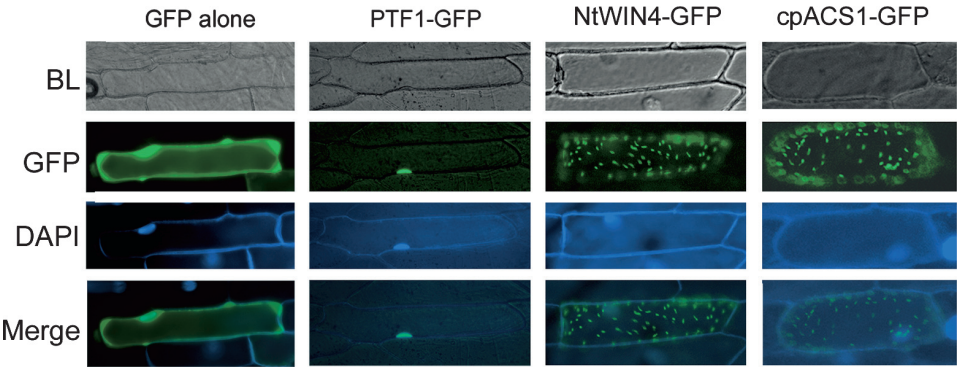

Figure 1. Comparison of sub-cellular localization. Onion epidermal cell layers were subjected to particle bombardment to introduce *GFP*, *AtPTF1-GFP*, *NtWIN4-GFP* or *cpACS1-GFP*. After incubation at 28°C for 6–12 h in the dark, samples were viewed under a microscope equipped with a fluorescence module. For DAPI staining, samples were incubated with 1 mg/ml DAPI solution before observation. Cells were observed for images under bright light (BL), for epifluorescence (GFP), for chromosomes (DAPI), and DAPI and GFP images were merged (Merge). Onion epidermal cell layers expressed GFP-tagged proteins (GFP), AtPTF1-GFP (PTF1-GFP), NtWIN4-GFP (NtWIN4-GFP). Cells expressing cpACS1-GFP (cpACS1-GFP) was used for the plastid control.

Table 1. Prediction of plastid localization within NtWIN4 and AtPTF1. NtWIN4 and AtPTF1 were examined by the TargetP 1.1 program (http://www.cbs.dtu.dk/services/TargetP/) using PLANT network without cutoffs. Examined samples were full length NtWIN4 (1–247), N-terminal-truncated NtWIN4 beginning from the second Met up to the basic domain (27–135) (Kodama and Sano 2006), full length *AtPTF1* (1–355), N-terminal region of AtPTF1 up to the basic domain (1–122), N-terminal-truncated full length AtPTF1 beginning from the second Met (58–355) and N-terminal-truncated AtPTF1 beginning from the second Met up to the basic domain (58–122). The probability scores of chloroplast (plastid) transit peptide (cTP), mitochondrial targeting peptide (mTP) and signal peptide for secretory pathway (SP) were estimated. Localization is predicted from the scores above.

Name	Position	Length	cTP	mTP	SP	Other	Localization
NtWIN4	1–247	247	0.183	0.107	0.121	0.671	Any other
	27–135	109	0.376	0.200	0.112	0.313	Chloroplast
AtPTF1	1–355	355	0.061	0.104	0.019	0.906	Any other
	1–122	122	0.071	0.092	0.021	0.901	Any other
	58–355	298	0.135	0.439	0.013	0.686	Any other
	58–122	65	0.207	0.524	0.032	0.541	Any other

control protein cysteine synthase, cpACS1-GFP (Figure 1, right panel). Previous *in silico* analysis with the localization prediction program, TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson et al. 2000) indicated that NtWIN4 beginning from the second Met at position 26 contained a putative plastid transit peptide, although the program failed to predict the transit signal in full length NtWIN4 beginning from the first Met (Kodama and Sano 2006) (Table 1). When AtPTF1 was examined by the same program, no plastid transit peptides were found in either full length or truncated peptide beginning from the second Met at position 58 (Table 1). Signals to other compartments, such as mitochondria and secretory pathways, were not found either (Table 1). Overall, biochemical and computer analyses indicated the low probability of AtPTF1 to localize in plastid.

Functions of AtPTF1 were further analyzed using nuclear transcription assays *in vivo* with the dual-luciferase assay. The constructs for this assay are shown as schematic illustrations in Figure 2A. When effector constructs of *GAL4BD* control and *GAL4BD-AtPTF1* were co-bombarded with a reporter plasmid and a reference plasmid into Arabidopsis MM2d cells, the *GAL4BD-AtPTF1* effector construct caused down-regulation of the luciferase activity to a level only 1/10 that of *GAL4BD* (Figure 2B). Thus AtPTF1 protein possesses transcriptional repression activity in nucleus, suggesting function as a nuclear transcriptional repressor *in vivo*.

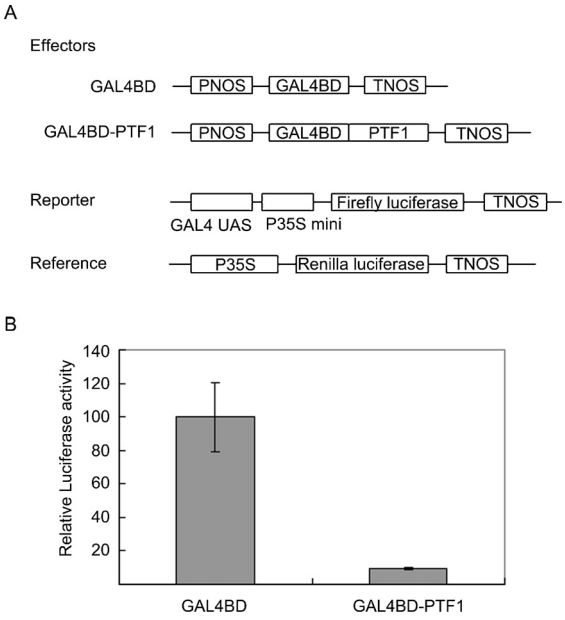

Figure 2. Nuclear transcription activity of AtPTF1. (A) Schematic illustration of plasmids used in the transcription assay. NosP, nopaline synthase promoter; NosT, nopaline synthase terminator; 35S, cauliflower mosaic virus (CaMV) 35S promoter; GAL4UAS, GAL4 binding sequence; 35Smini, CaMV35S minimal promoter. (B) Nuclear transcription activity assay. Effector constructs encoding GAL4BD-PTF1 were co-bombarded with the reporter and reference plasmids into 7-day old Arabidopsis MM2d cells. GAL4BD was used as the effector control. Luciferase activity of each transformant was normalized to the respective R-luciferase activity, calculated by dividing the luciferase activity of each clone by that of the clone containing the GAL4BD effector construct. Data are from triplicate experiments with standard deviations.

The full length polypeptide of NtWIN4 was predicted to be 28 kDa, but immunoblot analysis of intact proteins in plastids revealed a value of 17 kDa (Kodama and Sano 2006). Since the DNA binding domain in the bHLH motif was deleted in this form, it is likely that loss of the capacity for binding to DNA occurs. In contrast, AtPTF1 was initially identified as a DNA binding protein by the yeast one-hybrid screening method, and shown to recognize the ACC repeat region of LRP sequence to regulate LRP transcription in plastids (Baba et al. 2001). An AtPTF1-deficient mutant, *ptf1*, showed reduced activity of *psbD* LRP under continuous light conditions, and early bleaching, late flowering and dwarfism under short-day conditions. Based on these findings, AtPTF1 is proposed to regulate *psbD* LRP through its transcription in plastids.

Due to its unique features, AtPTF1 has often been referred to as an example of non-nuclear localized bHLH protein (Nagashima et al. 2004; Tsunoyama et al. 2004; Hanaoka et al. 2003; Sekine et al. 2002; Thum et al. 2001; Kanamaru et al. 2001). However, findings have

been rather controversial as to its function and localization. For example, *psbD* LRP was shown to be transcribed by a nuclear-encoded transcription factor, AtSig5 (Tsunoyama et al. 2004). This was confirmed with a mutant, *sig5*, in which *psbD* LRP activity was completely eliminated, whereas in *ptf1* mutants the activity was not affected (Baba et al. 2001). AtPTF1 is unlikely to be involved in light signaling based on the finding that light-dependent *psbD* transcription was not diminished in AtPTF1-deficient mutants (Tsunoyama et al. 2004).

Overall, above-mentioned genetic and our present biochemical studies pointed to that AtPTF1 might be a nuclear transcriptional repressor. To confirm this idea, identification of native AtPTF1 and its localization *in planta* is prerequisite by, for example, immuno-staining. Accordingly we are currently attempting to raise anti-AtPTF1 antibodies, despite of the reported difficulties perhaps due to a low yield of recombinant AtPTF1 proteins (Baba et al. 2001). Future studies with such antibodies will determine its size and cellular localization.

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