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Recombinant AtNF-YA, a Subunit of the *Arabidopsis* CCAAT-Binding Transcription Factor, Forms a Protein-DNA Complex with Mammalian NF-YB and NF-YC

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The evolutionarily conserved CCAAT binding protein NF-Y is a common regulatory DNA binding protein consisting of three distinct subunits. Unlike yeast and mammals, in which only a single copy of each subunit is encoded, *Arabidopsis* encodes a multi-gene family for each subunit in its genome. Compared with the NF-Y of mammals or yeast, very little is known about plant NF-Y homologs. Here *Arabidopsis* NF-YA subunits were isolated to determine whether they could form a heterotrimeric NF-Y complex with mammalian NF-YB and NF-YC. This resultant chimeric NF-Y complex had DNA binding ability to the same CCAAT sequences as those of the other life systems. Therefore, it is possible that plant NF-Y homologs might have biochemical characteristics similar to mammalian NF-Y, thereby suggesting its functional conservation among organisms.

Keywords: Arabidopsis genome, electrophoretic mobility shift assay, Nuclear Factor Y, yeast three-hybrid assay

Nuclear Factor Y (NF-Y), also known as CCAAT binding factor (CBF) and CP1, or HAP in yeast, is an evolutionarily conserved multimeric protein complex that specifically interacts with the CCAAT box to regulate gene expression (Maity and de Crombrugghe, 1998; Mantovani, 1999). The CCAAT box, one of the most common regulatory elements, is located between -60 and -100 b from the translation start site in eukaryotic promoters (Bucher, 1990; Mantovani, 1998).

Mammalian NF-Y is composed of three subunits – NF-YA (CBF-B), NF-YB (CBF-A), and NF-YC (CBF-C) – which correspond to yeast HAP2, HAP3, and HAP5, respectively (Becker et al., 1991; Maity et al., 1992; Maity and de Crombrugghe, 1998; Mantovani, 1999). An additional HAP4 subunit in yeast contains a highly acidic C-terminal domain that is characteristic of many transcriptional activators. This domain strongly activates transcription from the LexA operator when fused to the DNA binding domain of the LexA protein (Forsburg and Guarente, 1989; Olesen and Guarente, 1990). Mammalian NF-Y does not have a HAP4 homolog. Instead, glutamine-rich transcriptional activation domains are incorporated into the NF-YA and NF-YC subunits (Coustry et al., 1996).

The assembly pathway for the NF-Y complex has been revealed through *in vitro* protein-protein interaction assays and cross-linking. NF-YB and NF-YC subunits first interact with each other to form a heterodimer, thereby offering a new protein surface that enables interaction with the NF-YB subunit to form a heterotrimeric complex. All three subunits are required for DNA binding (Sinha et al., 1995, 1996; Kim et al., 1996; Liang and Maity, 1998).

Functional core domains of each subunit in the NF-Y complex have been identified in studies utilizing deletion and substitution mutants of the NF-Y subunit. These core domains are essential and sufficient for interacting with other subunits and binding to DNA (Olesen and Guarente, 1990; Maity and de Crombrugghe, 1992; Xing et al., 1993; Mantovani et al., 1994; Kim et al., 1996; Sinha et al., 1996; McNabb et al., 1997). Being highly conserved throughout evolution, they allow the subunits from different species to be functionally interchangeable (Li et al., 1992; Maity and de Crombrugghe, 1992; Kim et al., 1996; Sinha et al., 1996; McNabb et al., 1997). For example, yeast cell extracts lacking either a HAP2 or HAP3 subunit gain DNA-binding activity when supplemented by human NF-YA (HAP2) or NF-YB (HAP3), respectively, while, *in vitro*, the NF-YC (HAP5) from rats can interact with HAP2 and HAP5 subunits from yeast to form a CCAAT-binding protein complex (Chodosh et al., 1988; Sinha et al., 1995). Furthermore, the human HAP2 homolog (NF-YA) is able to complement the defect of the yeast *hap2* mutant, suggesting that the human subunit can substitute for its yeast counterpart *in vivo* (Becker et al., 1991). Thus, the essential core domains of NF-Y subunits from different organisms can interact and efficiently form a functional hybrid NF-Y complex.

Compared with the information available for NF-Y in yeast or mammals, very little is known about plant NF-Y proteins. So far, gene families encoding each subunit have been reported in *Arabidopsis*, and have been designated as AtNF-YA, AtNF-YB, and AtNF-YC families (Edwards et al., 1998; Gusmaroli et al., 2001, 2002). Unlike in yeast, no HAP4 homolog has been identified in plants. Moreover, the transcriptional activation domain of plant NF-Y remains unknown. Thus, it is unclear whether the *Arabidopsis* NF-Y consists solely of three subunits or whether a fourth subunit exists. All plant HAP homologs share an evolutionarily conserved functional domain with yeast and mammalian HAP homologs. The existence of a protein binding CCAAT or CAAT has also been described from plant nuclear extracts (sKusnetsov et al., 1999; Gusmaroli et al., 2001), and an *Arabidopsis* NF-YA subunit is known to suppress a *hap2* mutation from yeast (Edwards et al., 1998). Although these reports strongly support the idea that plant NF-Y homologs are also capable of forming an NF-Y complex, no direct evidence has been found of a plant NF-Y homolog being part of that complex. Therefore, to better understand the functioning of a plant NF-Y, the objective of this study was to investigate whether *Arabidopsis* NF-YA subunits can form a complex with mammalian NF-YB/NF-YC, and whether such a chimeric complex has CCAAT DNA sequence binding ability.

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MATERIALS AND METHODS

Plant Material

Plants of *Arabidopsis thaliana* ecotype Wassilewskija (Ws-O) were grown in soil under constant light at 22°C.

Preparation of DNA Constructs

cDNAs for AtNF-YA1 and YA2 were synthesized from *Arabidopsis* total RNA. For the yeast three-hybrid assay, the ORF of AtNF-YA was cloned into pJG4-5 (Clontech, USA) to generate a B42 activation domain (AD) fusion protein. The ORF of rat NF-YC was cloned into pEG202 (Clontech) to generate a LexA DNA binding domain (BD) fusion protein and the ORF of rat NF-YB was cloned into pLADR. After sequence confirmation by DNA sequencing, the DNA constructs were transformed into the *Escherichia coli* EGY048 (*Δhap2, Δhap3, Δhap5*) strain. For the electrophoretic mobility shift assay, the ORFs of AtNF-YA and rat NF-YC were cloned into pGEX (Pharmacia Biotech, USA) to create glutathione S-transferase (GST) fusion proteins. The ORF of rat NF-YB was cloned into pET-23 (Novagen, USA) to generate a histidine-tagged protein. Sequences of cDNAs obtained by PCR were verified by DNA sequencing. Synthesis of the recombinant protein was induced in *E. coli* BL21 (DE3) pLysS strain by adding 1 mM isopropyl β-D-thiogalactopyranosidase (IPTG).

Preparation of Recombinant Proteins

After induction with 1 mM IPTG, cells were pelleted and sonicated in lysis buffer containing 50 mM Tris-HCl (pH 7.9), 200 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, and 1 mM phenylmethylsulfonyl fluoride. Following centrifugation, the supernatant was loaded over glutathione-agarose beads (Pierce, USA), and GST-tagged proteins were eluted by 10 mM reduced glutathione (Sigma, USA). The extract containing the histidine-tagged protein was loaded onto a nickel-nitrilotriacetic acid-agarose affinity column and washed with buffer containing 50 mM Tris-HCl (pH 7.9), 50 mM NaH_2PO_4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 5% glycerol. The bound protein was then eluted with 0.5 M imidazole.

Electrophoretic Mobility Shift Assay (EMSA)

The purified, recombinant proteins were used for EMSA after extensive dialysis against 1X binding buffer (25 mM HEPES pH 8.0, 75 mM KCl, 10% glycerol, 1 mM EDTA, and 0.5 mM dithiothreitol). End-labeled oligonucleotides containing the CCAAT core region were used as a probe DNA. The recombinant NF-Y subunits were incubated with the labeled probe in a reaction buffer containing 25 mM HEPES (pH 8.0), 75 mM KCl, 10% glycerol, 1 mM EDTA, 0.5 mM dithiothreitol, 100 ng μL^{-1} of poly(dI-dC), and 1 μg of bovine serum albumin at room temperature for 20 min. The reactions were then fractionated on a 5% polyacrylamide gel in 0.5X TBE buffer at 4°C, and the labeled probes were detected by autoradiography.

Yeast Three-Hybrid Assay

Following protocol from the Yeast Protocol Handbook (Clontech), yeast transformants were selected on appropriate SD media that lacked specific amino acid nutrients. They were then shifted to media containing galactose and raffinose as carbon sources to induce protein expression from the *GAL1* promoter. β-Galactosidase activity was measured from three independent transformants by a liquid culture assay, using ONPG (o-nitrophenyl β-D-galactopyranoside) as substrate.

RESULTS

Arabidopsis Carries Multi-Gene Family of HAP Homologs

Unlike yeast and mammals, which contain single copies of NF-Y subunits, each plant NF-Y homolog exists as a multigene family in *Arabidopsis*. So far, 10 genes each for AtNF-YA and AtNF-YB and 9 genes for AtNF-YC have been reported in that genus (Gusmaroli et al., 2002). Here, the comparison of amino acid sequences from the AtNF-YA family and NF-YAs from other organisms revealed two conserved domains (Fig. 1) that have been proven essential to NF-YA functioning in yeast and mammals (Olesen and Guarente, 1990). All AtNF-YA amino acid sequences were compared with the PHYLIP program package, and a phylogenic tree was generated by comparative alignment of the conserved domains from 10 AtNF-YA genes (Fig. 2). Several subgroups could be identified with this AtNF-YA tree. For example, AtNF-YA2 formed a subgroup with AtNF-YA10, and AtNF-YA1 formed a subgroup with AtNF-YA9. The yeast NF-YA homolog, HAP2, and rat NF-YA were located outside of the major group. Two distantly related genes in that tree – AtNF-YA1 and AtNF-YA2 – were chosen for further analyses.

Arabidopsis NF-YA Can Interact with Mammalian NF-YB/NF-YC Heterodimer

In mammals, the NF-Y complex consists of three different

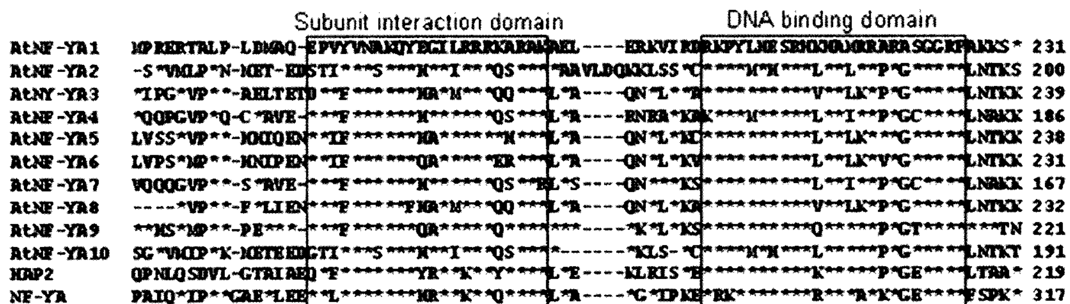

Figure 1. Amino acid sequence alignments of subunit interaction domains and DNA binding domains of NF-YA from *Arabidopsis* (AtNF-YA), yeast (HAP2), and mammals (NF-YA). Sequences were aligned using ClustalW program. Dashes represent gaps in sequences; * indicates identity with AtNF-YA1.

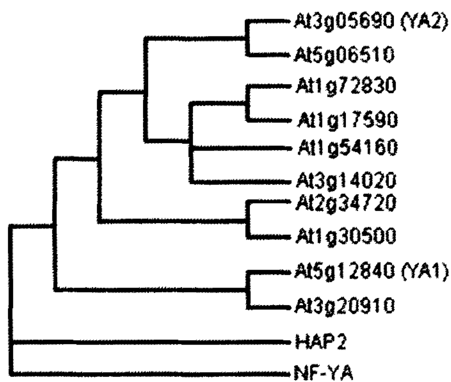

Figure 2. Phylogenetic tree of *Arabidopsis* NF-YA subunit gene family. Multiple sequence alignments were performed using ClustalX program. Tree was constructed based on amino acid sequences of AtNF-YA conserved domains shown in Figure 1.

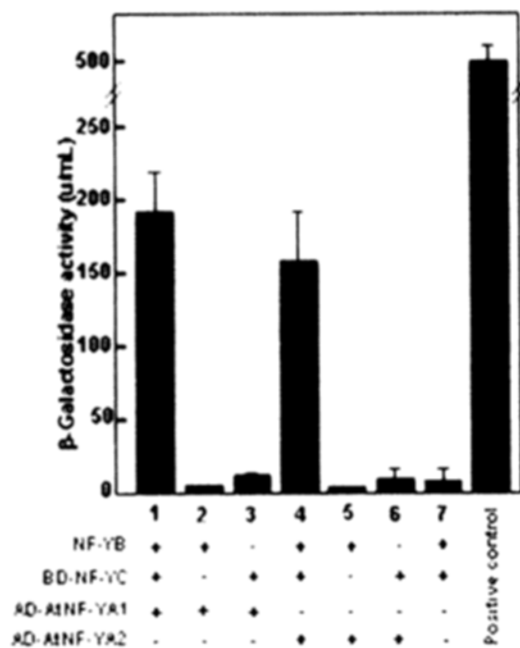

Figure 3. *Arabidopsis* NF-YA forms heterotrimeric complex with mammalian NF-YB/NF-YC subunits. Yeast three-hybrid complex assembly assays show phylogenetically distantly related AtNF-YA members, AtNF-YA1 and -YA2, forming complex with mammalian NF-YB and -YC in yeast. Quantification of β-galactosidase activity using colorimetric assays demonstrated complex formation of AtNF-YA and mammalian NF-YB and -YC (1st and 4th columns). No direct interactions between AtNF-YA and mammalian NF-YB or NF-YC were detected. Interaction between NF-YC and NF-YB was tested as a negative control (7th column). pSH17-4 was used as a positive control (8th column). Bars indicate standard deviation.

subunits, NF-YA, -YB, and -YC. Therefore, yeast three-hybrid assays were performed to examine the complex formation activity of *Arabidopsis* NF-YA homologs with mammalian NF-YB and -YC subunits (Fig. 3). NF-YC was cloned in the frame downstream of the LexA DNA binding domain (BD), while the AtNF-YA genes were cloned in the frame downstream of the B42 activation domain (AD). The interaction of subunits in yeast was measured by β-galactosidase activity. To test the interaction between AtNF-YA and NF-YB, LexA BD fused with NF-YB was used. Here, AtNF-YA and NF-YB did not interact directly without NF-YC (Fig. 3, 2nd and 5th columns), and no direct interaction was observed between AtNF-YA and NF-YC (Fig. 3, 3rd and 6th columns). Only the yeast lines expressing three subunits together were able to activate *lacZ* transcription, as indicated by β-galactosidase activity. Thus, interaction was required between all three subunits for reporter gene activation. These results demonstrate that AtNF-YA behaves like NF-YA from yeast and mammals. Interestingly, both AtNF-YA1 and YA2 interacted with mammalian NF-YB and NF-YC to form a complex, without any significant difference in their effectiveness. Although not all possible *Arabidopsis* NF-Y subunits were tested here, these data suggest that the interaction between *Arabidopsis* NF-Y subunits and mammalian NF-Y subunits most likely is not constrained. *Arabidopsis* NF-YA genes possibly act as a subunit of the NF-Y complex, and plants also can have a NF-Y complex consisting of at least three subunits.

Chimeric NF-Y Complexes Have DNA Binding Activity *in Vitro*

An NF-Y complex binds to the CCAAT sequence in the promoter region of target genes, such as for CYC1, MHC class II Ea, and the α2(I) collagen promoter in yeast, humans, and rats, respectively (Dorn et al., 1987; Karsenty et al., 1988). To determine whether these chimeric NF-Y complexes have DNA binding abilities, binding activities of the chimeric NF-Y to DNA sequences containing CCAAT from yeast (UAS2 of the CYC1) and mammals [Y-box of the MHC class II Ea and mCCAAT of the α2(I) collagen] were investigated. Recombinant subunits were produced in bacteria as GST- (AtNF-YA and NF-YC) and hexahistidine (NF-YB)-tagged fusion proteins. The electro mobility shift assay, using the recombinant AtNF-YA and NF-YB/YC dimer, revealed that the chimeric NF-Y complex bound the CCAAT sequences from yeast and mammals (Fig. 4A). Likewise, the mammalian NF-Y complex bound all the CCAAT sequences, without any significant difference in those binding activities (Fig. 4B). A competition assay with an unlabeled specific competitor and a nonspecific competitor that lacked CCAAT suggested that the binding was specific. Neither AtNF-YA alone nor the NF-YB/YC dimer without AtNF-YA were able to bind the CCAAT sequences (data not shown). These results demonstrate that the chimeric NF-Y containing AtNF-YA had DNA binding ability to both yeast and mammalian CCAAT sequences, strongly implying that *Arabidopsis* NF-Y homologs may possibly use the CCAAT motif as a binding site to regulate target genes in plants.

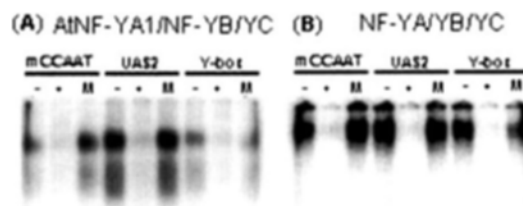

Figure 4. EMSA using chimeric NF-Y complex (**A**) and mammalian NF-Y (**B**). CCAAT sequences derived from yeast CYC1 (UAS2), human MHC class II Ea (Y-box), and rat a2(I) collagen (mCCAAT) upstream region were used as probe DNAs (–). Competition with 200-fold excess of unlabeled specific competitor (+), and lack of competition with 200-fold excess of unlabeled oligonucleotide without CCAAT motif (M) showed specific binding of complex.

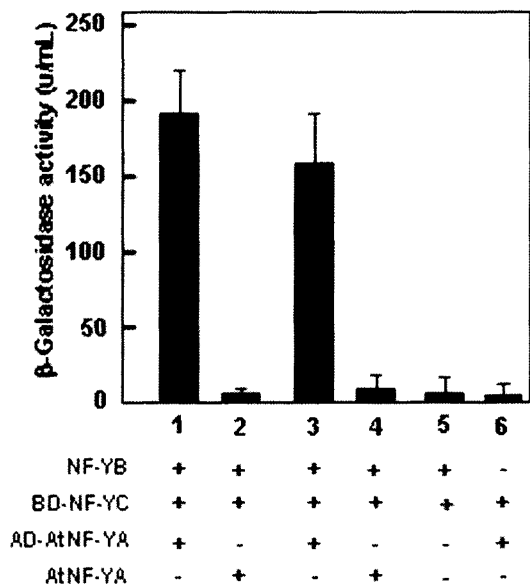

Figure 5. Chimeric NF-Y complexes do not have transcriptional activation function in yeast. Both AtNF-YA1 (1st column) and AtNF-YA2 (3rd column) interact with mammalian NF-YB and NF-YC to form ternary complex. However, modified yeast three-hybrid complex assembly assays performed without B42 activation domain demonstrates that neither AtNF-YA1 (2nd column) nor AtNF-YA2 (4th column) induces β-galactosidase activity. Interactions between NF-YB and NF-YC fused with BD, and between AtNF-YA1 fused with AD and NF-YC fused with BD, serve as negative controls (5th and 6th columns, respectively). Bars indicate standard deviation.

Chimeric NF-Y Complexes Show No Transcriptional Activation Functioning *in Vivo*

To investigate if the chimeric NF-Y complex had a transcriptional activation function, modified yeast three-hybrid assays were performed, using subunits that lacked the B42 activation domain. Chimeric NF-Y complexes that had the domain were able to activate transcription of the reporter gene (Fig. 5, 1st and 3rd columns). However, without the domain, the chimeric complex was unable to induce β-galactosidase activity (Fig. 5, 2nd and 4th columns). This result suggests that, unlike their mammalian homologs, the AtNF-YA subunits did not possess a transcription activation domain. Mammalian NF-YA has a glutamine-rich domain that is responsible for transcriptional activation. However, no conventional transcriptional activation domain is known in AtNF-YA. Therefore, the plant NF-Y complex may have a transcriptional activation mechanism different from that of mammals. The yeast HAP complex has a fourth subunit that is not required for DNA binding but which serves as a transcriptional activation domain in the complex (Olesen, 1990). Although no obvious HAP4 homologs exist in the *Arabidopsis* genome, the possibility that a fourth factor acts as the transcriptional activation domain in plants cannot be excluded. However, it may be that plant NF-Y does not require such an activation domain for its functioning. In fact, deletion of the transcriptional activation domain from even the mammalian NF-Y complex does not affect its ability to activate transcription (Hu et al., 2002).

DISCUSSION

Unlike yeast and mammalian NF-Y subunits, whose biochemical characteristics have been studied extensively, their plant homologs are not well characterized. Although the *Arabidopsis* genome encodes the gene families of NF-YA, -YB, and -YC homologs, a functional plant NF-Y complex has not yet been described. To understand the function of these plant homologs, phylogenetically divergent members of the AtNF-YA subunits were isolated, and interactions between the *Arabidopsis* NF-YA subunits and mammalian NF-YB/NF-YC subunits were studied. AtNF-YA1 and AtNF-YA2 interacted with the mammalian NF-YB and NF-YC subunits to form NF-Y complexes, without significant differences in their complex formations, as defined by β-galactosidase activity (Fig. 3, 1st and 4th columns). The plant NF-Y subunits probably follow the known mammalian NF-Y assembly pathway, in which the NF-YA subunit does not directly interact with either NF-YA or NF-YC subunits. Instead, dimerization of the NF-YB/NF-YC subunits is required for creating a new protein surface that interacts with the NF-YA subunit to form a trimeric NF-Y complex (Sinha et al., 1995). AtNF-YA did not directly interact with NF-YC (Fig. 3, 3rd and 6th columns). Neither did the yeast two-hybrid assay of AtNF-YA and -YB reveal any direct interaction between them (Fig. 3). Although not all AtNF-YA subunits were tested, because it is likely that they behave differently in plant cells, it would be logical to assume that at least some degree of interaction exists between plant NF-Y and mammalian NF-Y subunits. All *Arabidopsis* NF-Y subunits share highly conserved domains with NF-Y subunits from other organisms (Gusmaroli et al., 2001, 2002). These evolutionarily conserved domains are essential for NF-Y functioning, which leads one to propose that NF-Y may have functional similarity in different organisms.

NF-Y complexes bind the CCAAT motif in yeast and mammals (Dorn et al., 1987; Karsenty et al., 1988). Interestingly, the current study also showed that plant NF-YA allowed the formation of a complex between the mammalian NF-YB and -YC subunits and the CCAAT-containing DNA fragments from yeast and mammals (Fig. 4A). The fact that the chimeric NF-Y bound the same CCAAT as that of yeast and mammalian NF-Ys suggests the likelihood that the DNA binding ability of NF-Y to the CCAAT motif is well conserved throughout those kingdoms. This is the first report describing such interactions between plant NF-Y homologs and mammalian NF-Y subunits, as well as the formation of a functional NF-Y complex. Based on these results, one can conclude that the plant NF-Y subunits possess biochemical properties similar to the mammalian NF-Y subunits, and that they function similarly to NF-Y complexes from other organisms.

Data from the transcriptional activation test in yeast suggested that, unlike with mammals, plant NF-YA subunits do not possess a transcriptional activation domain (Fig. 5). In the former system, NF-YA has a glutamine-rich transcriptional activation domain at the C-terminal region, in contrast to *Arabidopsis* NF-YA homologs, which have no such obvious domains (Coustry et al., 1995, 1996). Moreover, the yeast HAP complex has an additional subunit HAP4 that is not required for DNA binding but which serves as a transcriptional activation domain for its functioning (Olesen, 1990). Although no obvious HAP4 homologs have been found yet in the *Arabidopsis* genome, one cannot exclude the possibility that an, as yet, unknown fourth subunit functions as the transcriptional activator for plant NF-Y. However, it is also possible that plant NF-Ys may not even require a

transcriptional activation domain but may, instead, function as a negative regulator or recruit other factors to promote transcriptional activity. In mammals, deletion of the transcriptional activation domain does not affect transcriptional activation of the target gene (Hu et al., 2002). Alternatively, plant NF-Ys may serve to recruit proteins with other functions, such as histone acetyltransferase to promoters, as occurs in mammals (Jin and Scotto, 1998; Caretti et al., 1999).

Data from this study leads one to propose that the plant NF-Y possibly acts in a manner similar to that of mammalian NF-Y, as demonstrated by the AtNF-YA subunit forming a functional ternary protein complex with mammalian NF-YB/NF-YC subunits *in vivo* and *in vitro*. However, further studies of their formation and DNA binding sequences in the plant NF-Y complex will be necessary to understand their regulatory mechanisms, which will then explain the functional differences and/or similarities between plant and mammalian NF-Ys.

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