Interactions and CCAAT-Binding of Arabidopsis thaliana NF-Y Subunits

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Abstract

Background: NF-Y is a transcription factor that recognizes with high specificity and affinity the widespread CCAAT box promoter element. It is formed by three subunits: NF-YA and the NF-YB/NF-YC heterodimer containing histone fold domains (HFDs). We previously identified a large NF-Y gene family in Arabidopsis thaliana, composed of 29 members, and characterized their expression patterns in various plant tissues.

Methods: We used yeast Two-hybrids assays (Y2H), pull-down and Electrophoretic Mobility Shift Assay (EMSA) in vitro experiments with recombinant proteins to dissect AtNF-YB/AtNF-YC interactions and DNA-binding with different AtNF-YAs.

Results: Consistent with robust conservation within HFDs, we show that heterodimerization is possible among all histone-like subunits, including the divergent and related LEC1/AtNF-YB9 and L1L/AtNF-YB6 required for embryo development. DNA-binding to a consensus CCAAT box was investigated with specific AtNF-YB/AtNF-YC combinations and observed with some, but not all AtNF-YA subunits.

Conclusions: Our results highlight (i) the conserved heterodimerization capacity of AtNF-Y histone-like subunits, and (ii) the different affinities of AtNF-YAs for the CCAAT sequence. Because of the general expansion of NF-Y genes in plants, these results most likely apply to other species.

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Introduction

The CCAAT box is one of the most ubiquitous promoter elements, being present in many, if not most of eukaryotic promoters [1]. Typically, it is found between −60 and −100 base-pairs from the transcriptional start site. The functional importance of the evolutionarily conserved consensus pentanucleotide has been widely established in several experimental systems. Twenty years of biochemical and genetic analyses have clarified that NF-Y [HAP2/3/5 in yeast] is a trimeric protein complex composed of NF-YA [HAP2], NF-YB [HAP3] and NF-YC [HAP5]. All subunits are required for DNA-binding and conserved throughout evolution [2]. NF-YB/NF-YC belong to the class of Histone Fold Domain (HFD) proteins, forming a tight dimer, structurally similar to H2A/H2B, with DNA-binding interaction modules [3]. Heterodimerization results in the formation of a surface for NF-YA association, allowing the resulting trimer to bind DNA with high specificity and affinity. The fungi HAP complex activates transcription through an additional subunit, HAP4, containing an acidic activation domain [4,5], unlike the mammalian NF-YA and NF-YC subunits which display large domains rich in Glutamines with transcriptional activation potential [6,7]. In plants, NF-Y also consists of three subunits and we and others have identified and classified them in Arabidopsis [8–10], and other species [11–15]. In general, plants have large families of genes, differentially expressed in various tissues: typically, 4–6 members are abundant and ubiquitous, while the others are restricted to certain tissues or developmental stages.

Genetic experiments were initially described for LEAFY COTYLEDON 1 (LEC1, AtNF-YB9) which has a role in embryo maturation and specification of cotyledon identity, with a unique pattern of expression confined to embryos ([16–18], reviewed in [19]). A LEC1 related member, L1L/AtNF-YB6, was shown to be able to partially complement the lec1 defect [20], and chimeric constructs demonstrated that the HFD domain is necessary and sufficient for LEC1 function in embryos [17]. The LEC1 homologues have similar roles in carrot [21,22] and Theobroma cacao [23]. Genetic analysis of AtNF-YA5 mutants indicate that it is involved in both ABA and blue light responses, together with LEC1 [24], and in drought resistance [25], similarly to AtNF-YB1 and YB2 and maize ZmNF-YB2 [26,27]. AtNF-YB2 and AtNF-
undertook Y2H assays, work. To clarify the stunning complexity of this system, we expected, given the wealth of previous biochemical and genetic advancements. The presence of 29 bona fide NF-Y genes in the Arabidopsis genome could potentially result in the formation of >900 alternative heterotrimetric combinations with different DNA-binding capabilities: the most obvious questions are whether there is specificity in interactions and whether all combinations are capable to bind to the CCAAT box. DNA-binding has been scored with carrot LEC1, one cNF-Y and two eNF-YCs [33], with OsHAP3A (NF-YB), six OsHAP5s (NF-YC) and one OsHAP2 [13], and AtNF-YB2 and AtNF-YB3 coupled to yeast HAP2 and HAP3 subunits [30]. A recent systematic study of Arabidopsis NF-Y subunits using Y2H assays reached the following conclusions [34]: (i) the HFD subunits do not homodimerize, (ii) they heterodimerize among them, with a notable degree of specificity, and (iii) AtNF-YAs can only bind to HFD dimers, and not to single subunits. The last point was expected, given the wealth of previous biochemical and genetic work. To clarify the stunning complexity of this system, we undertook Y2H assays, in vivo pull-down and Electrophoresis Mobility Shift Assay (EMSA), reporting the interaction map and DNA-binding activity of 24 members of the Arabidopsis NF-Y gene family.

**Results**

**Yeast Two-Hybrids assays**

Since NF-YB and NF-YC are known to form a tight heterodimer, whose interaction generates an optimal surface for NF-YA association, we used Y2H assays to systematically dissect the ability of each member of the AtNF-YB and AtNF-YC family to interact with each other. The bait and prey vectors contained the GAL4 DNA-binding domain (DBD) and GAL4 activation domain (AD), respectively. For each pair of AtNF-YB/AtNF-YC constructs, the Yeast Two-Hybrid interactions were tested in both configurations, to minimize the possibility of false positive and negative results. For both NF-Y family genes, we used the full length cDNAs corresponding to all AtNF-YB and AtNF-YC genes previously classified [9]. Three readouts were considered: His, Ade and LacZ, each driven by a different promoter under the control of the GAL4 responsive elements. Figure 1A shows the results of the different combinations with AtNF-YCs fused to the GAL4 DBD, and AtNF-YBs to GAL4 AD. On the other hand, Figure 1B shows the result obtained with AtNF-YCs fused to the GAL4 activation domain and AtNF-YBs to GAL4 DBD. Note that, in both cases, 3-AT was added to the yeast medium to minimize the growth due to self-activation. A first result is that the vast majority of the NF-YB and NF-YC family members can interact with each other in this in vivo assay. The only exception to this general observation is LEC1/AtNF-YB9, which does not interact significantly with any of the AtNF-YCs, in both configurations (Fig. 1), except for a suboptimal interaction with AtNF-YC3 and only with the AD configuration (Fig. 1A). A weaker interaction can be observed between specific pairs, like AtNF-YB2/AtNF-YC6 and AtNF-YB3/AtNF-YC7, in both configurations. Other pairs with suboptimal affinity are AtNF-YB2/AtNF-YC2, AtNF-YB3/AtNF-YC2, AtNF-YB3/AtNF-YC6, AtNF-YB1/AtNF-YC7 and AtNF-YC3/AtNF-YB10 (Fig. 1B). To further confirm these interactions and better quantify their strength, liquid Y2H Assays were performed by measuring β-GAL activity under conditions of exponential growth. For the liquid assay, we used the AtNF-YB (DBD) and AtNF-YC (AD) configurations shown in Figure 1B. The results of these experiments are shown in Figure 2. As previously determined by in plate assays, the liquid assay confirmed that LEC1/AtNF-YB9 (DBD) does not significantly interact above background levels with any AtNF-YC subunits. The liquid assay confirmed the weak interactions detected by the in plate assay. Furthermore, it was possible to detect a couple of additional weak interactions between AtNF-YB7/AtNF-YC6 and AtNF-YB10/AtNF-YC6. On the other hand, AtNF-YB1, AtNF-YB5 and AtNF-YB6, and to a lesser degree AtNF-YB2, showed robust interactions with all AtNF-YC family members. Overall, this set of experiments indicate that the vast majority of the HFD combinations heterodimerize, with very few specific exceptions.

**In vitro analysis**

The negativity of LEC1/AtNF-YB9, unable to interact with any AtNF-YC, and the positivity of L1L/AtNF-YB6, which binds to all partners, are not expected. To substantiate the Y2H assays, we produced and purified recombinant proteins, as well as in vitro produced proteins by transcription and translation [TriT] of different subunits (Fig. S1). We chose AtNF-YB2 and AtNF-YA6 because they are rather “conventional” structure-wise when compared to the mammalian homologues. AtNF-YC were mixed with an excess of His-tagged recombinant AtNF-YB2 and loaded on Ni2+ saturated Nickel columns. Figure 3 shows the results of such experiments. As expected, control columns did not retain any AtNF-YC subunit in the bound fractions in the absence of AtNF-YB2 (Fig. 3A and 3B, lanes 5). On the other hand, all AtNF-YCs were bound, with varying degrees of efficiency, in the presence of AtNF-YB2 (Fig. 3A and 3B, lanes 3), or L1L/AtNF-YB6 (Data not shown). While this assay is not quantitative, it does confirm that the two AtNF-YBs are able to retain on the column all AtNF-YCs, consistent with the results obtained by the Y2H assay. In the same assay, AtNF-YA6 was also retained with different AtNF-YC combinations when His-tagged AtNF-YB2 was added (Fig. 3B), indicating that interactions are observed in the presence of the three subunits.

Having shown that most HFD subunits are able to interact both in vivo and in vitro, the next relevant question concerns the affinity of combinations for the CCAAT box. To answer this question, recombinant proteins were produced by TriT and used in EMSAs with a consensus, high affinity NF-Y oligonucleotide [1]. In Figure 4, several members of the Arabidopsis subunits were first assayed in the presence of the mouse NF-YA/NF-YC heterodimer. As negative controls we used the mouse dimers as an additional negative control (Fig. 4A, lane 1). Recombinant mouse NF-YA alone was also used as negative control (Fig. 4C, lane 2). Positive controls were the mouse dimeric combinations alone (Fig. 4A lane 2, Fig. 4B lane 1 and Fig. 4C lane 4). In vitro transcribed and translated Luciferase was added to the same mouse dimers as an additional negative control (Fig. 4A, lane 1). Recombinant mouse NF-YA alone was also used as negative control (Fig. 4C, lane 2). Positive controls were the mouse recombinant NF-YY trimer (Fig. 4C, lane 1), and single mouse NF-Y subunits added to the corresponding mouse dimeric combinations: NF-YB to NF-YA/NF-YC (Fig. 4A, lane 9), NF-YC to NF-YA/NF-YB (Fig. 4B, lane 11) and NF-YA to NF-YB/NF-YC (Fig. 4C, lane 11). Surprisingly, none of the AtNF-YBs added to the mouse NF-YA/NF-YC led to the formation of a complex with an electrophoretic activity different from the negative controls (Fig. 4A). In the case of the AtNF-YCs, instead, all subunits generated a discrete band with mouse NF-YA/NF-YB, with mobility somewhat similar to that of mouse NF-Y: the bands were weak for AtNF-YC3, AtNF-YC7 and AtNF-YC8, but quite robust for the other six AtNF-YCs tested. For AtNF-YAs, AtNF-YA2 and AtNF-YA4 were negative, whereas AtNF-YA3, AtNF-YA6, AtNF-YA8 and AtNF-YA9 were all capable of generating bands with mobilities similar to mouse NF-Y. These results indicate that the majority of the AtNF-YA and AtNF-YC members behave as
canonical NF-Ys, as they associate with mouse subunits and bind to the CCAAT box.

The negativity of the AtNF-YBs in the TnT-EMSA assays (Fig. 4A) was troubling: therefore, we decided to investigate whether this was an artefact due to the use of mouse recombinant NF-YA and NF-YC subunits and/or to the TnT system used. First, we selected two AtNF-YBs -AtNF-YB2 and L1L/AtNF-YB6- which are proficient in interactions with all AtNF-YCs according to the Y2H assay. We produced and purified single His-tagged AtNF-YB2 and AtNF-YB6 recombinant proteins in E. coli, together with two AtNF-YC subunits, namely AtNF-YC3 and AtNF-YC7 (Fig. S1). The choice of these members were driven by two types of considerations, the first being expression patterns, the second relatedness to mouse subunits: AtNF-YB2 and AtNF-YC3 are the most ubiquitously expressed and less “variant”, whereas L1L/AtNF-YB6 and AtNF-YC7 are strictly tissue-specific and the most deviant. The HFD proteins were found in inclusion bodies, as expected, denatured and efficiently renatured when mixed together [35,36]. In one set of experiments, to the Arabidopsis NF-YB/NF-YC dimers we added recombinant AtNF-YA6, one of the AtNF-YAs positive in the EMSAs with mouse subunits (Fig. 5A). The Arabidopsis NF-YB/NF-YC dimers were also added to recombinant mouse NF-YA (Fig. 5A). AtNF-YA6 is able to generate NF-Y-like bands when AtNF-YC3 dimerized with either AtNF-YB2 or AtNF-YB6; the AtNF-YC7 combinations, on the other hand, yielded either no band or a smeary pattern. The same was essentially observed with mouse NF-YA (Fig. 5A), except that the AtNF-YB6/AtNF-YC3 combination was more efficient in binding, paralleling the efficiency of the mouse NF-Y trimer. The difference in mobilities of At-NF-YA6 and mouse NF-YA complexes are visible and most likely due to the different molecular mass of these two NF-YA proteins (308 and 347 residues, respectively). Again, the AtNF-YC7 combinations gave no band or a smear, indicating that heterotrimers with this subunit are very inefficient in CCAAT-binding. We decided to further dissect the DNA-binding activity of this heterodimer in the presence of other AtNF-YA family members: Figure 5B shows that an NF-Y complex was obtained with AtNF-YA3, AtNF-YA6, AtNF-YA8 and AtNF-YA9. Taken together, the results of Figure 5 are consistent with the set of experiments previously performed by

![Table](https://example.com/table.png)

**Figure 1. AtNF-YB-AtNF-YC interactions by colony yeast two hybrids assays.** A. The indicated AtNF-YCs were fused to the Activation Domain (AD) and tested with AtNF-YB fused to the DNA-binding domain of GAL4. B. Same as A, except that the reverse experiment was tested, namely the AtNF-YBs fused to the Activation Domain were matched to the AtNF-YCs fused to the DNA Binding Domain. ++ refers to robust growth on the selective medium, + weak growth, and − no growth.

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using mouse recombinant subunits (Fig. 4). These data indicate that AtNF-YA2 and AtNF-YA4 are either incapable to associate to AtNF-YB6/AtNF-YC3 and mouse HFD dimers or to bind to DNA.

L1L/AtNF-YB6 and LEC1/AtNF-YB9 belong to the same clade and are genetically linked. Having shown that L1L/AtNF-YB6 is capable to heterotrimerize and bind to CCAAT, we wondered whether the lack of heterodimerization of LEC1/AtNF-YB9 was due to some artefacts of the Y2H system. We decided to use an E. coli coexpression system in which the HFDs of either protein was coexpressed with the HFD of AtNF-YC3: Figure 6A shows that both heterodimers are produced and purified from soluble bacterial extracts. The copurification of (untagged) AtNF-YC3 with the His-tagged AtNF-YBs is a clear sign of heterodimerization. Surprisingly, when we expressed LEC1/AtNF-YB9 alone, rather than being confined to inclusion bodies, the protein was very efficiently produced in a soluble form, which is very unusual for HFD proteins. Next, we performed EMSAs with a CCAAT oligonucleotide, using the two heterodimers and mouse NF-YA: Figure 6B shows that both gave shifted bands, with mobilities similar to NF-Y. The affinities were lower with respect to the mouse NF-YB/NF-YC used as positive control, but similar among them. Note that in this particular experiments, we used the minimal heterotrimerization/DNA binding domain constructs consisting of the evolutionarily conserved regions of each subunit [3], with a 31 bp Cy5-labelled probe in Agarose-EMSA, which resulted in faster DNA-protein complexes. AtNF-YB9 alone did not show any DNA binding. Taken together, these data prove that LEC1/AtNF-YB9 can heterodimerize, trimerize with NF-YA and bind to CCAAT as efficiently as its closest relative, L1L/AtNF-YB6.

Discussion

One of the most pressing questions in biology is to understand the interactions of transcription factors among each other and with their natural DNA targets. As they have often evolved in complex families, whose members share some common features and diverge in others, the intricacies of the role of each member needs to be clarified. This is particularly challenging in plants, where genes encoding TFs have expanded to amazing numbers. One such example is NF-Y, the heteromeric CCAAT-binding protein, whose subunits are encoded by single copy genes in most eukaryotes, including mammals [2], while in *Arabidopsis* and other plants each is represented by large families. We began a systematic investigation of the interactions among 24 AtNF-Y subunits, by using Y2H *in vivo* and *in vitro* assays. Some of our data, notably those on LEC1/AtNF-YB9, indicate that negative results of Y2H assays should be confirmed by independent biochemical means, before interactions can be ruled out.

Dimerization

By and large, the *Arabidopsis* NF-Y HFD subunits -AtNF-YBs and AtNF-YCs- are able to heterodimerize, whether by Y2H assays or by *in vitro* interactions. These results are not surprising, based on considerations made on the crystallographic structure of the mouse NF-YB/NF-YC dimer [3], and of similar HFD dimers, including H2A/H2B [35]. These analyses have revealed that the a2 helix of the HFD is the core of the dimerization surface, thanks
primarily to hydrophobic contacts. Another region of importance is the α1 helix, stabilized by hydrophobic interactions that are stacked against a conserved tryptophan at position 85 of α2 helix of NF-YC. Essentially all AtNF-YBs and AtNF-YCs have hydrophobic residues at appropriate positions, thus the widespread heterodimerizations we observed came to modest surprise. With respect to the Y2H experiments reported by Hackenberg et al. [34], as well as previous data [13,31], we note the following differences.

(i) Some clear preferences for heterodimer formation between specific AtNF-YBs and AtNF-YCs were observed in the present and in the Hackerberg studies: only AtNF-YC6 and AtNF-YC7 show a marked preference for selected AtNF-YB subunits, in our study; all AtNF-YCs, except AtNF-YC3 and AtNF-YC9, have clear preferences in the Hackenberg data. On the AtNF-YBs side, AtNF-YB2, AtNF-YB3, AtNF-YB4, AtNF-YB7 and AtNF-YB10 show reduced affinity for one, or sometimes two AtNF-YCs; the same applies in the reported study. Even with our knowledge of the structure, it is quite difficult to rationalize these preferences, which seems quantitative more than qualitative.

(ii) In the Hackenberg study, AtNF-YB11, B12, B13 and AtNF-YC10, 11, 13 are very selective, with a tendency to heterodimerize among them: the likeliest explanation is that these are not true NF-Y subunits, but rather resemble to other H2A/H2B-likes [KT, CT, RM, in preparation]: it should be remembered, in fact, that the TBP/TATA-binding NC2α/NC2β and the chromatin remodeling and DNA-Polymerase ε subunits Chrac15/Dpb3/Dpb4 share extended conservation and have highly similar structures.

Figure 3. AtNF-Y Subunits interactions in vitro. A. The indicated labelled, TnT produced NF-YCs were assayed in affinity assays with recombinant AtNF-YB2 containing an His-tag. Load (L), flow-through (FT) and bound (B) fractions of NTA Nickel columns, with (Lanes 2 and 3) and without (Lanes 4 and 5) His-AtNF-YB2 were run on SDS-PAGE gels and labelled proteins were revealed by autoradiography. B. Same as A, except that labelled, TnT produced AtNF-YA6 was added to the load fraction.

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We have previously reported that mammalian NF-YB/NF-YC do not cross heterodimerize with NC2 subunits [38], and there are structural reasons for this [3]. Kumimoto et al. have shown that AtNF-YB2 and B3 interact strongly with AtNF-YC3, C4 and C9 in Y2H and in genetic terms [31], which is in line with our data, but not with the Hackerberg study, in which they lack AtNF-YC4 binding. In rice, the homologue of AtNF-YB2 (OsHAP3A) interacts in Y2H with all OsHAP5s, including the homologue of AtNF-YC4, except for homologues of AtNF-YC2 and C3 [13].

AtNF-YC5, AtNF-YC7 and AtNF-YC8, which belongs to a common clade and are the most tissue-restricted members of the AtNF-YC [8], were negative for AtNF-YB heterodimerization in the Hackenberg et al., study, the former in both AD and DB combinations, the latters in one. In our data, these AtNF-YCs were generally positive for all AtNF-YBs, except AtNF-YC7, negative with AtNF-YB3 and AtNF-YB4.

**Figure 4. EMSAs of AtNF-Y subunits with mouse NF-Y.**

A. Electrophoretic Mobility Shift Assay of the indicated AtNF-YB with recombinant mouse NF-YA and NF-YC using a labeled CCAAT-containing oligonucleotide. B. Same as A, except that AtNF-YCs were used with recombinant mouse NF-YA and NF-YB. C. Same as A, except that AtNF-YA were used with recombinant mouse NF-YB and NF-YC. The migration of the mouse NF-Y complex is indicated.

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LEC1/AtNF-YB9 showed a dual behaviour in our hands: no interactions with AtNF-YCs in the Y2H assays, yet efficient heterodimerization with AtNF-YC3 by co-expression of the two proteins in E. coli. In the Hackenberg study, LEC1/AtNF-YB9 was positive with most AtNF-YCs; moreover, the carrot homologue of LEC1 was able to bind DNA in vitro with two NF-YC homologues [33]. Thus, in this specific case, our Y2H was clearly misleading.

All in all, different Y2H data show some discrepancies, most likely due to technicalities in the expression vectors, yeast productions or activation assays. We also have to bear in mind that yeast possesses endogenous HAPs (as well as NC2 and Dpb3/4), indeed shown to interact with some of the plant members [30], thus possibly influencing the results of such assays. Our experiments with LEC1/AtNF-YB9 illustrates the dangers of relying only on this assay in the case of negative results.

We are intrigued by the unusual capacity of LEC1/AtNF-YB9 to form homodimers and remain soluble in bacteria: to the best of our knowledge, this is unique among HFDs, which are normally found as inactive, precipitated proteins in inclusion bodies, when not overexpressed with the appropriate partner [36]. This brings up the question of whether LEC1 homodimers are formed in plants. We found that they do not bind DNA, most likely because of lack of interactions with NF-YA, which absolutely requires NF-YC. It is possible that there is regulation of homo- to heterodimer formation: for example, post-translational modifications (PTMs), not performed in bacteria, could be required to render the HFD prone to heterodimerization: these are histone-like proteins, and histones are crucially controlled by a wealth of PTMs, and we have recently obtained evidence that mouse NF-YB is modified a-la H2B (RM, in preparation).

LEC1/AtNF-YB9 and L1L/AtNF-YB6 are capable to efficiently heterodimerize with AtNF-YC3, trimerize and bind to DNA, and the latter also with all AtNF-YC partners in Y2H assays. These data fits with genetic experiments, which established that L1L complements the LEC1 mutants, and in domain swapping experiments with other AtNF-YBs, the B domain -corresponding to the HFD [40]- is required for complementation. In addition to the AtNF-YC and AtNF-YA partners, LEC1 and L1L could exert their roles through interacting proteins, such as MADS box OsMADS6 and OsMADS18 [41], Pirin1, an iron-containing member of the cupin superfamily involved in a pathway leading to an ABA-mediated delay in seed germination [24]. Additional proteins interacting with AtNF-Ys are bZIP67, interacting with AtNF-YC2 in the regulation of CRUCIFERIN C [CRC] and SUCROSE SYNTHASE2 [SUS2] in Arabidopsis protoplasts [21], and, most importantly, CONSTANS and CONSTANS-like proteins in Arabidopsis and tomato [42,43] involved in determining the proper flowering timing with specific members of AtNF-YBs and AtNF-YCs [31,44].

DNA-binding

The formation of NF-Y heterotrimers was tested with selected AtNF-YB/AtNF-YC HFD dimers. While the HFD dimer contributes substantially to DNA-binding, mostly through α1 helices, L1 and L2 loops, the subunit that confers the sequence-specificity is NF-YA. On the HFD side, the heterotrimerization surface relies in selected residues in the α2 helix of NF-YB and in the αC helix of NF-YC. The E90 and E98 of mouse NF-YB, important for NF-YA binding [45], are conserved in all AtNF-YBs [8–10,34]. The αC helix of AtNF-YC, on the other hand, shows differences in at least three members: AtNF-YC5 possesses an R at position 109 of mouse NF-YC, instead of a hydrophobic residue; AtNF-YC3 has two Aspartates at position 111 and 112, instead of hydrophobics, together with Isoleucine at position 113, instead of the helix capping Proline [3]; finally, AtNF-YC7 has a four aminoacids addition in the α3 helix, which extends it for an additional turn, hence displacing the LC domain and αC helix.
from their natural positions. Not coincidentally, these three members were not proficient in DNA-binding in our assays. Although the interaction with AtNF-YA6 appears to be visible with recombinant proteins, it remains to be seen whether other residues directly contacting DNA in L1 and L2 loops (N86 in AtNF-YC5 and G113 in AtNF-YC7, instead of a conserved Lysine) might explain the decrease in DNA affinity of this group of AtNF-YCs.

It was initially troubling to obtain negative results in EMSAs with the TnT-produced AtNF-YBs, but this was most likely due to technical problems of the translation extract, possibly inhibiting trimerization, or production of inactive AtNF-YBs in the absence of coexpression of AtNF-YCs: in fact, recombinant AtNF-YBs produced from *E. coli*, including the divergent LEC1/AtNF-YB9 and L1L/AtNF-YB6, were positive in DNA-binding. Interestingly, mutation of an Aspartate at position 55 of LEC1 is sufficient to abrogate LEC1 function *in vivo* [17]. D55 is located at the beginning of the α2 Helix, in a region that lies on the surface of the dimer: most other *Arabidopsis* and mammalian NF-YBs have a Lysine, conserved in H2B, and predicted to be involved in protein-DNA interactions [3]. L1L/AtNF-YB6 also has an Aspartate at this position, which might be considered as a “signature” for this subfamily: the change might decrease affinity for DNA, but an important result in our study is that it certainly does not abolish it: in essence, no AtNF-YB is “deviant” enough to have lost the DNA-binding capacity.

On the NF-YA side, the evolutionarily conserved domain is responsible for trimerization and CCAAT-binding. Protein-
protein interaction assays and EMSAs indicate that the majority of AtNF-YAs are able to interact with AtNF-YB2/AtNF-YC3 and L1/L3/AtNF-YB6/AtNF-YC3. Indeed, they are quite proficient in association to the mouse NF-YB/NF-YC dimer. In particular, the AtNF-YA6 shows robust CCAAT binding, which strongly suggests that AtNF-YA5, not tested here, behaves similarly: the two belong to a common clade, and the DNA-binding subdomain is absolutely conserved. AtNF-YA5 is so far the only AtNF-YA for which genetic experiments were reported: mutation causes drought stress and overexpression drought resistance [25]; our data suggest that the mechanisms are related to prototypical CCAAT-binding.

Only AtNF-YA2 and AtNF-YA4 were negative, suggesting that they are either incapable to trimmerize or bind DNA. Note that AtNF-YA7 and AtNF-YA10 not tested here might behave similarly, since the residues required for subunits interactions and DNA-binding are identical to AtNF-YA4 and AtNF-YA2, respectively. Several papers described two separate 20 aminooacid stretches as required for subunits interactions and DNA-binding [46–48]. Detailed mutagenesis of the mouse and yeast subunits pinpointed several aminooacids necessary for the two functions. In the subunits interaction domain, no dramatic changes are observed, and indeed important residues are conserved in AtNF-YA2 and AtNF-YA4, with the notable exception of R273 (mouse), which is G147 in AtNF-YA2 and G137 in AtNF-YA4: potentially, this could affect trimmerization, since an R to G mutation in yeast HAP2 does decrease the efficiency of HFP association significantly [46]. We note, however, that in none of the other AtNF-YAs, nor in most other plant NF-Y genes, there is an Arginine at this position: in proficient members of the family tested here, an Alanine is present. Most importantly, AtNF-YA2 and AtNF-YA4 were previously tested for heterotrimerization, and indeed showed efficient association with HFDs [34]: in all likelihood, therefore, they have decreased DNA-binding affinity, despite an overall conservation of key DNA-binding residues. Can we take these data as an indication that some of the AtNF-YAs have lost the capacity to bind DNA? If it is indeed so, what might be their function? The most obvious answer is that if they do bind NF-YB/NF-YC subunits interaction domain fusions (pBD) and pDEST22 for activation domain fusions (pAD). The pDEST22 and pDEST22 vectors were transformed into Saccharomyces cerevisiae strain Yp69-4A (trp1-901 leu2-3, 112 ura3-52 his3-200 gal4A gal80A lys2::GAL1-HIS3 GAL2::ADE2 met2::GAL7-lacZ) [49]. Yeast Two-Hybrid assay was performed as described below.

Yeast Two-Hybrid (Y2H) analysis

Haploid Yeast α and A were transformed respectively with pBD and pAD vector constructs using the lithium acetate method [47] and selected on Yeast Synthetic Dropout (YSD) medium lacking Leu and Trp, respectively. Yeast carrying pBD vectors were tested for autoactivation on selective medium with 3-bromo-l-hloro-3-indolyl-b-D-galactopyranoside (X-Gal), on medium lacking histidine and supplemented with different concentrations of 3-amino triazole (0, 3, 5, 10, 25 and 50 mM) and on medium lacking adenine. Mating type α and A were mated and diploids selected on YSD medium lacking Leu and Trp.

Two-hybrid interactions were assayed on selective YSD medium lacking Leu, Trp, and Ade or His supplemented with 30 mM 3-amino triazole. Selection was performed at 28°C for 4 days.

Liquid Two-Hybrid Assay

Semi-quantitative assay for comparing the strength of AtNF-YB and AtNF-YC subunits interactions was performed by liquid LacZ assay. For the liquid assay, we used the AtNF-YB (DBD) and AtNF-YC (AD) configuration.

Yeast was inoculated in selective medium and grown for 8–9 h, then centrifuged at 4500 rpm for 5 min. Pelletted cells were resuspended in 5 ml of selective medium and grown O/N at 28°C. Cells were centrifuged at 4500 rpm for 5 min and the pellet was resuspended in 0.5 ml of cold water, centrifuged again for 30 sec at 14000 rpm. Pellet was resuspended in 250 μl of pre-cooled Breaking Buffer (100 mM Tris-HCl pH 8.0, 10% Glycerol, 1 mM Dithiothreitol, protease inhibitors) and frozen in liquid nitrogen. Unfrozen samples were subjected to 10 cycles of vortex/ice with glass beads, centrifuged at 14000 rpm for 10 min and supernatant has been recovered. Then 20 μl of protein extract were transferred to a 1.5 ml centrifuge tube and added with 800 μl of Z-Buffer (60 mM NaH₂PO₄, 40 mM Na₂HPO₄-anhydrous, 10 mM KCl, 1 mM MgSO₄, 50 mM β-Mercaptoethanol) and 200 μl ortho-Nitrophenyl-β-galactosidase (ONPG) 4 mg/ml. The tube was incubated at 37°C until the solution became yellow, for a maximum of 45 min and the reaction was stopped adding 400 μl of 1.5 M Na₂CO₃. The samples were centrifuged for 30 sec at 13000 rpm and the optical density at 420 nm (OD₄₂₀) was determined. Activity in Miller Units was calculated according to the formula [OD₄₂₀ *1.4]/(0.0005*CV*V*t) where CV = concentration of protein extract (mg/ml); V = volume of protein extract (ml); t = time (min). Activity of AtNF-YB GAL4-DBD with GAL4-AD fused with no AtNF-YC subunit has been used as control.

Materials and Methods

Yeast strains and plasmid construction

The cDNAs corresponding to each AtNF-Y subunit used in the Yeast-Two Hybrid assay, were amplified from Arabidopsis cDNA libraries using gene specific primers containing the attB1 and attB2 sequences for homologous recombination and subsequently cloned into pDONOR201 vector (Life Technology). AtNF-YB and AtNF-YC coding sequences in pDONOR201 were subsequently cloned in the GAL4 Gateway vector system: pDEST32 for DNA binding domain fusions (pBD) and pDEST22 for activation domain fusions (pAD). The pDEST32 and pDEST22 vectors were transformed into Saccharomyces cerevisiae strain Yp69-4A (trp1-901 leu2-3, 112 ura3-52 his3-200 gal4A gal80A lys2::GAL1-HIS3 GAL2::ADE2 met2::GAL7-lacZ) [49]. Yeast Two-Hybrid assay was performed as described below.

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Production of recombinant AtNF-YB and generation of 35S-Labeled AtNF-YC

To examine the in vitro interaction between AtNF-YB and AtNF-YC subunits, His-tagged AtNF-YB and 35S labelled AtNF-YC were produced and used for pull-down experiments.

Chemically competent E. coli BL21 cells were transformed by thermal shock with 100 ng of pET32A or pET32B, in which AtNF-YB coding sequences were cloned. Transformed cells were inoculated in LB broth (5 ml) with ampicillin (100 ng/ml) at 37°C for 16 h. An aliquot (3 ml) of this culture was inoculated in 200 ml of the same medium and let grow until an OD600 of 0.6 was reached. The expression of each protein was induced with IPTG (1 mM) for 3 h.

Cells were harvested by centrifugation at 6000 rpm for 10 min at 4°C and suspended in Sonication Buffer (300 mM KCl, 20 mM Tris-HCl pH 7.8, 0.05% NP40, 1 mM EDTA, 1 mM PMSF, 5 mM β-Mercaptoethanol) containing a cocktail of Proteinase inhibitors (12.5 mg/ml leupeptin, 5 mg/ml pepstatin, 5 mg/ml chymostatin). The cells were then thoroughly disrupted with a sonicator (10 cycles, 20 sec each). The samples were centrifuged at 23000 rpm at 4°C for 90 min to separate supernatant (SN) from inclusion bodies (IB). The SN and IB (Resuspended in 100 mM KCl, 20 mM Tris-HCl pH 7.8, 5 mM β-Mercaptoethanol, 1 mM PMSF, 6 M GuCl) were loaded onto Nickel-Agarose columns (Sigma). After thoroughly washing with Washing Buffer (20 mM Tris-HCl pH 7.8 10% glycerol, 300/1000/100 mM KCl), the proteins bound to the columns were eluted in Elution Buffer (2 mM Tris-HCl pH 7.8, 10% glycerol, 100 mM KCl, 1 mM PMSF, 5 mM β-Mercaptoethanol, 300 mM Imidazol). Finally, eluted fractions from SN and IB were subjected to dialysis to remove Imidazol.

AtNF-YC subunits, cloned in pCR4TOPO (Invitrogen), were synthetized and 35S-labeled by coupled transcription and translation in 25 μl of nucleic-treated rabbit reticulocyte lysate (TnT, Promega).

His pull-down assay

His-tagged AtNF-YB recombinant proteins (500 ng) and 10 μl of AtNF-YCs produced by TnT were incubated together at 37°C for 30 min in 100 μl of NDB100 (20% glycerol, 100 mM KCl, 20 mM Tris-HCl pH 7.8, 0.5 mM EDTA, 5 mM β-Mercaptoethanol). After incubation, recombinant proteins were loaded onto a Nickel-Agarose column (Sigma), incubated for 3 h at 4°C, and then centrifuged at 4000 rpm for 1 min at 4°C to recover the “flow through” (FT). After washing 3 times, they were eluted (“bound”, B) with 30 μl of Elution Buffer (NDB100 containing 5 mM β-Mercaptoethanol, 0.25 M imidazol, PIC 1x). As negative controls, aliquots (10 μl) of the same AtNF-YC subunits produced by TnT were incubated with the Nickel-Agarose column. We did not observe any specifically bound AtNF-YC subunits in the negative controls performed in the absence of His-tagged AtNF-YB. One third of FT and B samples were subjected to SDS-PAGE, transferred to a nitrocellulose membrane (150 mA/gel, 1.5 h), and analyzed by Western blotting using anti-His antibodies; the remaining two thirds of each sample were analysed by autoradiography to detect AtNF-YC subunits.

HFD heterodimer Protein expression and purification

The 6His-AtNF-YB/AtNF-YC soluble HFD dimers were purified exploiting the T7-driven co-expression system described in [3,50]. AtNF-YC3 (AA 55–148) (corresponding to the HFD region of mouse NF-YC AA 27–120) was subcloned in the pmncYC vector; LEC1/AtNF-YB9 (AA 56–148) or L1L/AtNF-YB6 (AA 26–118) subunits (corresponding to mouse NF-YB HDF AA 49–141) were subcloned in pET15b, resulting in 6His-N-terminal fusions. 6His-LEC1/AtNF-YB9, or 6His-L1L/AtNF-YB6, was expressed in E. coli BL21(DE3) together with, or not, AtNF-YC3, and purified by Ni-chelate affinity chromatography (HiSelect, Sigma-Aldrich), as described in [3], in buffer A (10 mM Tris pH 8.0, 400 mM NaCl, 2 mM MgCl2, 5 mM imidazole), and eluted by subsequent additions of 1 bed volume of buffer B containing 100 mM Imidazole. Indicated 6His-HFD protein purification eluates were dialyzed against buffer B (10 mM Tris pH 8.0, 400 mM NaCl, 2 mM DTT) containing 10% glycerol, and used in Fluorescence Agarose gel EMSAs. The soluble NF-Y heterotrimetric subunit complex and 6His-NF-YA were produced as described in [50], and purified by Ni-chelate affinity chromatography (HiSelect, Sigma-Aldrich) in buffer A, followed by thrombin cleavage of the NF-YA C-terminal His-tag, and gel filtration (GF) chromatography (HiLoad Superdex75, Amersham Pharmacia) in buffer B. GF fractions corresponding to the NF-Y heterotrimer, or the NF-YA isolated subunit, were collected, and used in Fluorescence Agarose gel EMSAs, after addition of 10% glycerol for storage.

Electrophoretic Mobility Shift Assays

For electrophoretic mobility shift assays 32P labelled fragments (~10000 CPMs- are incubated in NF-Y Buffer (20 mM Hepes pH 7.9, 50 mM NaCl, 5% Glycerol, 5 mM MgCl2, 5 mM β-ME) with the recombinant proteins (1–5 ng), in a total volume of 10 μl; after incubation for 15’ at 20°C, we added 2 μl of 1 x NF-Y buffer containing Bromphenol Blue and samples loaded on a 4.5% Polyacrylamide in 0.5 x TBE. Gels were dried and exposed. For Fluorescence Agarose Gel EMSAs of Figure 6, heterotrimer formation and CCAAT-box DNA-binding of the 6His-AtNF-YB/AtNF-YC soluble dimers was assayed with Cy5-labeled oligos, by addition of GF purified mouse NF-YA (AA 233–303). Equal protein amounts of Ni-purified 6His-AtNF-YB/AtNF-YC HFD dimers (5, 6, or 9 ng/ul) were mixed in 15 μl reactions with the 5’-labeled 31 bp oligo probe derived from human HSP70 promoter CCAAT box sequence (Cy5-CTTCTGAGCCCAAT-CAACCGAGTCGATGAGGC) in DNA binding mix (20 mM ds oligo, 20 mM Tris pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 5 mM MgCl2, 2.5 mM DTT, 0.1 mg/ml BSA, 5% glycerol), in the presence of 40 nM NF-YA, where indicated. Ni-purified mouse 6His-NF-YB/AtNF-YC (1, 3, 6 ng) were used in positive controls. After 30 min incubation at 23°C, binding reactions were loaded on a 2.5% agarose gel and separated by electrophoresis in 0.5 x TBE. Fluorescence gel images were obtained with a Typhoon 8610 Variable Mode Imager (Molecular Dynamics).

Supporting Information

Figure S1 TnT and recombinant proteins production.

A. AtNF-YA, AtNF-YB and AtNF-YC subunits were synthesized and 35S-labeled by coupled transcription and translation in nucleic-treated rabbit reticulocyte lysate (TnT, Promega). B. His-tagged AtNF-YA6, AtNF-YB2 and AtNF-YB6, AtNF-YC3 and AtNF-YC7 have been produced in E. coli and purified by Nickel-Agarose columns (Sigma). Load (L), flow-through (FT), wash (W) and eluted (E) fractions of NTA Nickel columns are shown.

(TIF)
Author Contributions
Conceived and designed the experiments: RM CT. Performed the experiments: VC BT GG ML NG KP. Analyzed the data: RM CT NG.

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