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RESEARCH ARTICLE

NUCLEAR FACTOR Y, Subunit A (NF-YA) Proteins Positively Regulate Flowering and Act Through *FLOWERING LOCUS T*

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Abstract

Photoperiod dependent flowering is one of several mechanisms used by plants to initiate the developmental transition from vegetative growth to reproductive growth. The NUCLEAR FACTOR Y (NF-Y) transcription factors are heterotrimeric complexes composed of NF-YA and histone-fold domain (HFD) containing NF-YB/NF-YC, that initiate photoperiod-dependent flowering by cooperatively interacting with CONSTANS (CO) to drive the expression of *FLOWERING LOCUS T* (*FT*). This involves NF-Y and CO binding at distal *CCAAT* and proximal "CORE" elements, respectively, in the *FT* promoter. While this is well established for the HFD subunits, there remains some question over the potential role of NF-YA as either positive or negative regulators of this process. Here we provide strong support, in the form of genetic and biochemical analyses, that NF-YA, in complex with NF-YB/NF-YC proteins, can directly bind the distal *CCAAT* box in the *FT* promoter and are positive regulators of flowering in an *FT*-dependent manner.

Author Summary

For plants to have reproductive success, they must time their flowering with the most beneficial biotic and abiotic environmental conditions—after all, reproductive success would likely be low if flowers developed when pollinators were not present or freezing temperatures were on the horizon. Proper timing mechanisms for flowering vary significantly between different species, but can be connected to a variety of environmental cues, including water availability, temperature, and day length. Numerous labs have studied the molecular aspects of these timing mechanisms and discovered that many of these pathways converge on the gene *FLOWERING LOCUS T* (*FT*). This means that understanding precisely how this gene is regulated can teach us a lot about many plant species in both natural and agricultural settings. In the current study, we focus on day length as an essential cue for flowering in the plant species *Arabidopsis thaliana*. We further unravel the

complexity of *FT* regulation by clarifying the roles of *NUCLEAR FACTOR Y* genes in day length perception.

Introduction

Plants undergo numerous developmental phase changes that are both species specific and intimately linked to the environments in which they evolved. One of the most important phase changes—as evidenced by the numerous pathways controlling the process—is the transition from vegetative to reproductive growth (recently reviewed in [1]). For many plant species, a potent trigger of the transition to reproductive growth is photoperiod-dependent flowering. Photoperiod-dependent species use the relative length of day and night to either activate or repress flowering such that it is timed with the appropriate environmental conditions to maximize reproductive success.

The model plant *Arabidopsis thaliana* (Arabidopsis) is a so-called long day plant; that is, it flowers rapidly when days are longer than ~12 hrs [2–5]. Central to measuring photoperiod is the circadian regulation of *CONSTANS* (*CO*) transcription and the light-mediated regulation of CO protein accumulation [6]. CO protein is stabilized in long days and is able to bind and transcriptionally activate *FLOWERING LOCUS T* (*FT*) [7, 8]. FT protein is the principal mobile hormone—or "florigen"—that travels from leaves, where the photoperiod signal is perceived, to the shoot apex, where the floral transition occurs [9–12]. In the shoot apex, *FT* activates its downstream targets, which includes *APETALA 1* (*AP1*) and *SUPPRESSOR OF CONSTANS 1* (*SOC1*) [13, 14]. Members of the heterotrimeric NUCLEAR FACTOR-Y (NF-Y) transcription factor family are required for activation of the *FT* promoter, thus initiating the downstream events leading to the floral transition [15–20].

NF-Y transcription factors are composed of three independent protein families, NF-YA, NF-YB, and NF-YC. To activate target genes, NF-YB and NF-YC dimerize in the cytoplasm and move to the nucleus where the heterodimer interacts with NF-YA to create the DNA-binding, heterotrimeric NF-Y transcription factor [21–24]. NF-Y binding is widely regarded as sequence specific to the evolutionarily conserved *CCAAT* motifs, with some modified sites having been reported [17, 25, 26]. All direct contacts with the pentanucleotide are made by NF-YA, while the NF-YB/NF-YC dimer primarily makes non-sequence specific contacts in adjacent regions, stabilizing the complex [27]. While ubiquitous to eukaryotes NF-Y subunits have undergone an extensive expansion in plants [28, 29]. For example, Arabidopsis has ten members of each *NF-Y* gene family [29].

Several NF-YB and NF-YC subunits have been demonstrated to regulate photoperiod dependent flowering [15, 18–20, 30, 31]. Briefly, *nf-yb2 nf-yb3* double and *nf-yc3 nf-yc4 nf-yc9* triple mutants flower very late under normally inductive photoperiods [19]. In both cases, the single mutants have either no effect or comparatively mild effects on flowering time, indicating overlapping functions for these family members. NF-YB and NF-YC proteins can physically interact with CO and loss of function mutations lead to *FT* expression downregulation [15, 18–20, 30]. Finally, genetic and biochemical data suggest that NF-Y complexes bind the *FT* promoter at a distal *CCAAT* box (-5.3kb from start codon), while CO binds several clustered proximal **CO r**egulatory **e**lements (CORE—approx. -200bp upstream from start). Chromatin loops stabilize the interactions between these two distally separated, DNA-bound complexes [8, 16, 32, 33].

In light of HFD (Histone Fold Domain dimer; NF-YB/NF-YC) interactions with CO in photoperiod-dependent flowering, immediate questions are whether NF-YA proteins are

regulators of photoperiod-dependent flowering and whether this is CO-dependent and exerted through regulation of *FT*. Related to NF-YA roles in flowering, initial reports demonstrated that they can negatively regulate flowering as overexpression of some *NF-YA* genes caused late flowering [20, 34]. Because NF-YA and CO proteins share a region of sequence homology, one possibility is that they compete for occupancy on NF-YB/C dimers. In this scenario, NF-YA and CO might play opposing negative and positive roles, respectively. However, recent reports suggest a more complex scenario, given 1) Genetic evidence for the importance of the -5.3kb *FT CCAAT* box in flowering [16, 32, 33]; 2) DNA-bound mammalian NF-Y crystal structure showing that NF-YA makes the direct contacts with the *CCAAT* box and that CO contains key differences in amino acids necessary for these contacts [16, 27, 35, 36]; and 3) Both direct and indirect evidence that CO directly binds CORE sites on *FT* [7, 8].

Here we address the roles of NF-YA proteins in *FT* binding, expression regulation, and photoperiod-dependent flowering time. Using a combination of genetic and biochemical approaches, we show complete NF-Y complexes, including NF-YA, bound to the -5.3kb *FT CCAAT* box. We further demonstrate that NF-YA and NF-YB constructs that can drive early flowering, and perform this activity in an *FT*-dependent manner. Collectively our data indicate that NF-YA acts as a positive regulators of flowering and that *FT* is a key regulatory target of NF-Y/CO complexes in the photoperiod-dependent flowering pathway.

Results

NF-YA genes can be positive regulators of photoperiod dependent flowering

To identify NF-YAs involved in flowering, we first examined constitutive overexpression (35S promoter) in first generation (T1) transgenic plant lines for each of the 10 Arabidopsis *NF-YA* genes (lines described in [37]). We observed that *p35S:NF-YA2* and *p35S:NF-YA6* expressing plants consistently flowered earlier than Col-0. Nevertheless, confident interpretations of these data were complicated by the pleiotropic, dwarf phenotypes in most overexpressing lines. In fact, lines that constitutively overexpressed *NF-YA6* were infertile and did not survive (as previously described, [37]). We were able to isolate and quantify stable, third generation transgenic *p35S:NF-YA2* lines and compare them to several other stable lines for constitutively expressed *NF-YA* genes (Fig 1A). Two independent *p35S:NF-YA2* lines flowered early (~10 leaves, compared to 13 for wild type Col-0 plants), while overexpression of other *NF-YA* genes either did not alter flowering or actually caused modestly later flowering. This is consistent with the original observations of Wenkel *et al.*, 2006 [20]. We note that all of these plant lines showed similar dwarf phenotypes, suggesting that our flowering time observations were not directly correlated with this phenotype.

To avoid the pleiotropic effects from ectopically overexpressing *NF-YA2*, we additionally generated stable, native promoter transgenic plant lines (*pA2:NF-YA2*). Presumably due to position effects, some of these lines had high relative transcript abundance of *NF-YA2* (~60 fold overexpressed) and were early flowering (Fig 1B, S1 Fig). Interestingly, these plants appeared phenotypically normal, suggesting that the dwarf phenotypes of *p35S*-driven lines is more related to ectopic expression than overexpression, *per se*. Note that our previous research on *NF-Y:GUS* expression patterns showed that both *NF-YA2* and *NF-YA6* had very strong vascular expression, consistent with the expected localization of floral promoting genes (Fig 1C and [19, 32, 33, 35, 38, 39]).

As discussed above, previous reports suggest that CO, NF-YB and NF-YC regulate flowering primarily by controlling *FT* expression which, in turn, rapidly upregulates *AP1* [18, 19, 30, 32, 33, 40–42]. We used the stable *pNF-YA2:NF-YA2-1* plant line to test if *NF-YA2* regulates

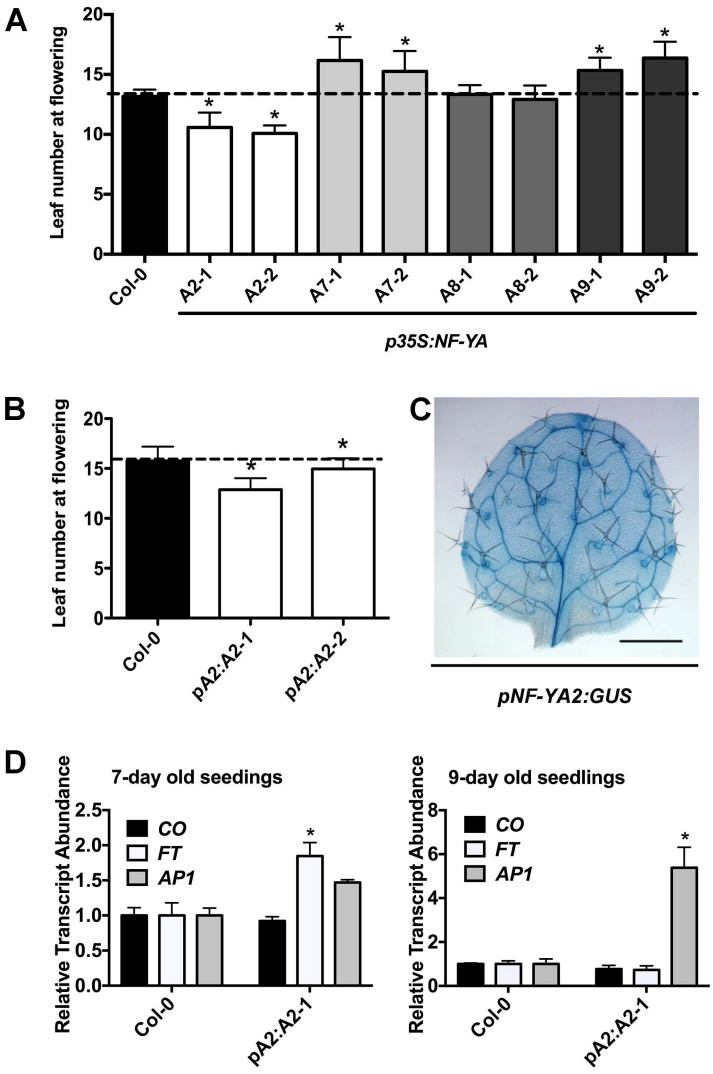

Fig 1. NF-YA2 is a positive regulator of photoperiod dependent flowering. A) Flowering time quantification of two independent plant lines each (plant lines 1 and 2) for *p35S:NF-YA2* (white bars), *p35S:NF-YA7* (light grey bars), *p35S:NF-YA8* (grey bars), and *p35S:NF-YA9* (dark grey bars) (n≥12/line). B) Flowering time quantification of two independent *pNF-YA2:NF-YA2* plant lines (n≥24). C) The expression pattern of *pNF-YA2:GUS* in leaves of 10 day old plants. D) Relative transcript abundance of *CO*, *FT*, and *AP1*. Asterisks in 1A and 1B represent significant differences derived from one-way ANOVA ($P < 0.05$) followed by Dunnett's multiple comparison post hoc tests against Col-0. Asterisks in 1D represent significant differences derived from Student's T-tests ($P < 0.05$). All experiments were repeated with identical results.

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the same set of genes. We used the time points of seven and nine days after germination because they correlate with the initiation of flowering signals in long day grown plants [38]. *NF-YB* and *NF-YC* do not affect the relative transcript abundance of *CO* [18, 19, 30]; likewise, *CO* was not misregulated in the *NF-YA2* overexpressor (Fig 1C). However, the relative transcript abundance of *FT* was upregulated in seven day old *pNF-YA2:NF-YA2-1* plants, which was followed by significant *AP1* upregulation by day nine. We did not see increased relative transcript abundance of *FT* in day 9, possibly due to negative feedback as downstream targets in the signaling cascade, such as *AP1*, are activated. We note that the moderate upregulation of *FT* transcript abundance due to *NF-YA2* overexpression is consistent with *NF-YB2*

overexpression [18]. These results suggest that *NF-YA2*, like its *NF-YB* and *NF-YC* counterparts, regulates flowering by controlling *FT* expression.

The NF-YB2^{E65R} mutation prevents NF-YA subunits from entering into NF-Y complexes

Because of the apparent difficulties in working directly with NF-YAs, the likely overlapping functionality between family members in flowering (e.g., Hou reports that *nf-ya2* mutants have no flowering delay, [17]), and lethality [43, 44], we decided to indirectly manipulate NF-YA function by altering its ability to interact with the HFD dimer. In mammals, the NF-YB^{E92R} mutant protein specifically loses interaction with NF-YA, but not NF-YC [22]. Crystal structure analysis of the NF-Y complex demonstrated that this glutamic acid makes multiple contacts with NF-YA Arg249 and Arg253 [27]. Alignments between human and Arabidopsis NF-YB proteins show that this glutamic acid (E65 in Arabidopsis NF-YB2) is completely conserved (Fig 2A) and examination of other published alignments also confirm this conservation in the monocot lineage [35, 45–47]. Thus, we reasoned that NF-YB2^{E65R} mutations would eliminate the ability of NF-YA to enter floral promoting NF-Y complexes and allow us to further test the hypothesis that NF-YA proteins are positive regulators of photoperiod-dependent flowering.

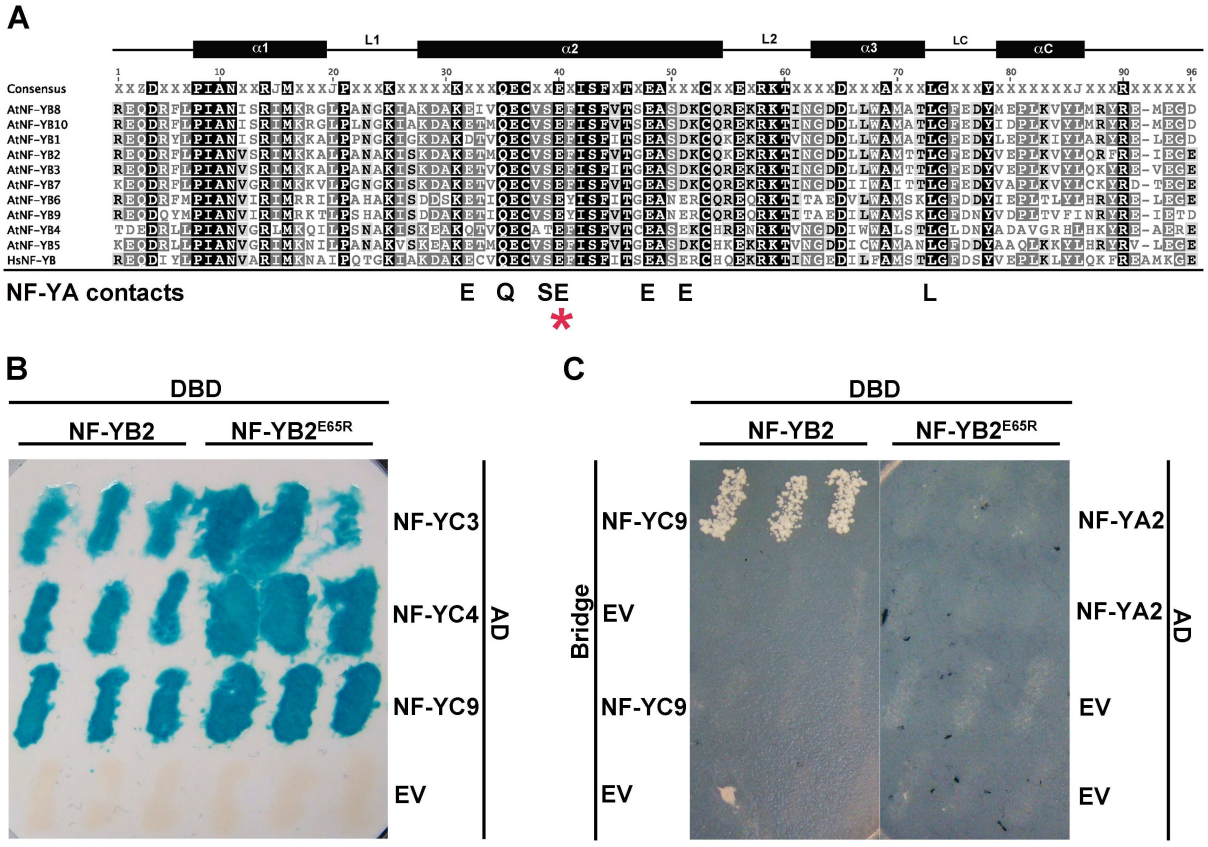

Fig 2. NF-YB2^{E65R} loses interaction with NF-YA subunits. A) Alignment of the core domain of human and Arabidopsis NF-YB subunits. * marks the position of the conserved glutamic acid required for interaction with NF-YA in humans [27]. B) NF-YB2 and NF-YB2^{E65R} interact with NF-YC3, NF-YC4, and NF-YC9 in Y2H assays. C) NF-YB2, but not NF-YB2^{E65R}, interacts with NF-YA2 when NF-YC9 is expressed using a bridge vector in yeast three-hybrid assays. DBD: DNA binding domain, AD: activation domain, EV: empty vector control.

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We first used yeast two hybrid assays to test if NF-YB2^{E65R} could interact with NF-YC3, NF-YC4, and NF-YC9 and found that both NF-YB2 and NF-YB2^{E65R} were able to physically interact with the NF-YCs (Fig 2B). Since NF-YA heterotrimerizes with HFD dimers and not individually with NF-YB or NF-YC [48], we used yeast three hybrid assays to test the ability of NF-YA2 to enter into a complex with NF-YB2^{E65R} and NF-YC9 (Fig 2C). As predicted, NF-YA2/NF-YB2/NF-YC9 complexes formed, but the NF-YB2^{E65R} variant prevented formation of the trimeric NF-Y complex. Thus, the NF-YB2^{E65R} provides a powerful genetic tool to test the requirement for NF-YA in photoperiod-dependent flowering.

The NF-YB2^{E65R} mutation prevents rescue of a late flowering *nf-yb2 nf-yb3* mutant

As previously described [16], here and throughout this study, we examined T1 plants as it gave a better representation of flowering time responses by eliminating bias associated with the selection of individual transgenes. For each transgene we examined 15–20 individual plants and for a few key lines we generated two independent T3 transgenic lines from individuals that were representative of the observed T1 average plant.

We predicted that *p35S:NF-YB2^{E65R}* would be unable to drive early flowering in wild type Col-0 or rescue the *nf-yb2 nf-yb3* late flowering phenotype. We tested this by overexpressing both *p35S:NF-YB2* and *p35S:NF-YB2^{E65R}* in each background and measuring flowering responses in 15–20 randomly selected T1 plants for each construct. We found that *p35S:NF-YB2* showed a trend towards earlier flowering in Col-0, but only caused significantly earlier flowering in a subset of independent experiments (Fig 3A, non-significant example shown). However, *p35S:NF-YB2 nf-yb2 nf-yb3* plants flowered ~20 leaves earlier than the parental mutant (Fig 3B–3D). With the *p35S:NF-YB2^{E65R}* version, Col-0 actually flowered significantly later than normal (likely indicating dominant interference with the endogenous complexes) and there was no rescue of the *nf-yb2 nf-yb3* late flowering phenotype (Fig 3A–3D).

To confirm that NF-YB^{E65R} was localizing properly, we compared plants expressing *NF-YB2-YFP* and *NF-YB2^{E65R}-YFP* and found that both had identical nuclear localization patterns (S2A Fig). Additionally, we measured NF-YB protein accumulation in late flowering *p35S:NF-YB2^{E65R}* T1 plants (all >31 leaves at flowering) compared to a well-characterized, stable, early flowering *p35S:NF-YB2* line (all proteins were translationally fused to the HA epitope). The *p35S:NF-YB2^{E65R}* T1 lines showed the expected variation in NF-YB protein accumulation; note that even lines that strongly accumulated NF-YB2^{E65R} could not rescue late flowering (S2B and S2C Fig; e.g., in S2B Fig compare protein accumulation in *p35S:NF-YB2^{E65R}* lines 6, 10, 11, and 12 to the stable p35S:NF-YB2 line). Stable, single insertion T3 lines, selected based on high NF-YB2^{E65R} protein accumulation, showed the same pattern of late flowering (Fig 3C and S2D Fig). Finally, we compared stable *p35S:NF-YB2 nf-yb2 nf-yb3* and *p35S:NF-YB2^{E65R} nf-yb2 nf-yb3* for relative transcript abundance of *NF-YB2*, *FT*, and *AP1* (Fig 3E). Although both lines had very high, ~equivalent *NF-YB2* transcript abundance, *p35S:NF-YB2* resulted in increased *FT* and *AP1* transcript abundance while *p35S:NF-YB2^{E65R}* significantly suppressed both. Collectively, this data shows that the *p35S:NF-YB2^{E65R}* plant lines are not able to drive the typical flowering responses associated with *NF-YB2* overexpression and a possible explanation for this would be the loss of interaction with NF-YA.

NF-YA2 and NF-YA6 heterotrimerize with NF-YB2 and NF-YC3 *in vitro* to bind the -5.3kb *CCAAT* box

We previously showed that NF-YB2 and NF-YC3, together with mouse NF-YA, are able to bind a 31bp, *CCAAT*-containing oligonucleotide from the *FT* -5.3kb site [16]. At that time we

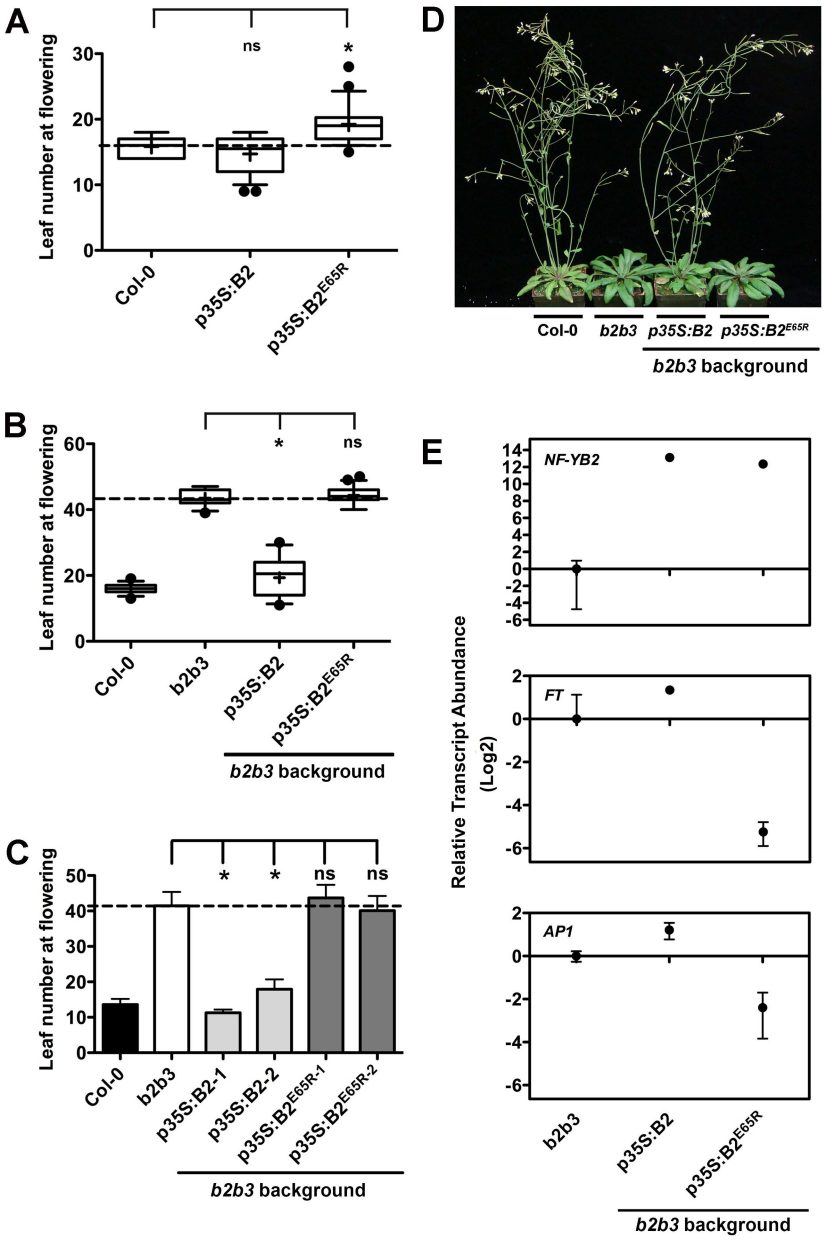

Fig 3. *p35S:NF-YB2^{E65R}* cannot rescue the *nf-yb2 nf-yb3* late flowering phenotype. A) Flowering time quantification of 15–20 randomly selected T1 *p35S:NF-YB2* and *p35S:NF-YB2^{E65R}* plants in the Col-0 background. B) Flowering time quantification of 15–20 randomly selected T1 *p35S:NF-YB2* and *p35S:NF-YB2^{E65R}* plants in the *nf-yb2 nf-yb3* background. C) Flowering time quantification of two independent, stable T3 *p35S:NF-YB2* and *p35S:NF-YB2^{E65R}* plant lines in the *nf-yb2 nf-yb3* background (n≥12). D) Representative plants of *p35S:NF-YB2* and *p35S:NF-YB2^{E65R}* in the *nf-yb2 nf-yb3* background. E) Relative transcript abundance of *NF-YB2*, *FT* and *AP1* in stable T3 *p35S:NF-YB2* and *p35S:NF-YB2^{E65R}* plants in the *nf-yb2 nf-yb3* background. Asterisks in 3A, 3B and 3C represent significant differences derived from one-way ANOVA ($P < 0.05$) followed by Dunnett's multiple comparison post hoc tests against *nf-yb2 nf-yb3*.

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were unsure of the likely Arabidopsis NF-YA(s) involved in flowering: with the data presented here and a recent publication [17] showing that NF-YA2 and NF-YA6 can act as positive regulators of flowering, we used EMSA to test if NF-YA2 and NF-YA6 are able to bind a probe encompassing the -5.3kb *CCAAT* box on *FT*. In the presence of NF-YB2/NF-YC3 dimers,

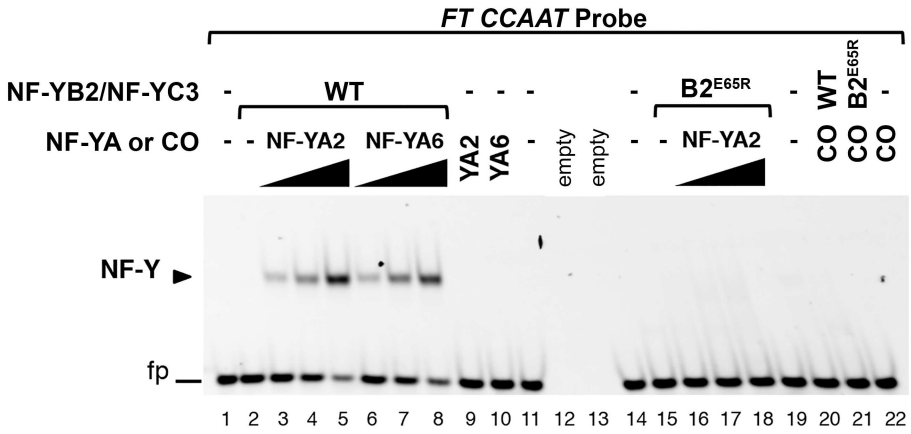

Fig 4. NF-YA2 and NF-YA6 bind the *FT*-5.3kb *CCAAT* box as a trimer with NF-YB2 and NF-YC3. NF-Y trimerization and *FT CCAAT* binding was assessed by EMSA analysis. An *FT CCAAT* probe was incubated with wild type (WT, lanes 2–8; 20) or E65R mutant ($B2^{E65R}$, lanes 15–18; 21) NF-YB2/NF-YC3 dimers (60 nM) in the presence of NF-YA2 (lanes 3–5; 16–18), or NF-YA6 (lanes 6–8) at increasing molar ratios (3, 4.5 or 6 fold), or CO (lanes 20, 21; 6 fold molar ratio). As controls, NF-YA2 (lane 9), NF-YA6 (lane 10), or CO (lane 22) were incubated alone with the probe, at the highest concentration of the dose curve (360 nM), in the absence of NF-YB2/NF-YC3. Lanes 1, 11, 14, 19: probe alone, without protein additions; lanes 12, 13: empty lanes. The NF-Y/DNA complex is indicated by a labelled arrowhead. fp: free probe.

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NF-YA2 and NF-YA6 bound the *CCAAT* probe in a concentration-specific manner (Fig 4). However, neither NF-YA2 nor NF-YA6 could individually bind the *CCAAT* probe. Further, CO did not bind the *CCAAT* probe, individually or in the presence of the NF-YB2/NF-YC3 dimer (Fig 4 and S3 Fig). We additionally tested equivalent concentrations of NF-YA2 with NF-$YB2^{E65R}$/NF-YC3 and found that this combination completely lost the ability to bind the *CCAAT* probe. This data demonstrates that plant NF-Y complexes interact with and bind the *FT* -5.3kb *CCAAT* box in a manner that is similar, if not identical, to their mammalian counterparts.

p35S:NF-YB2^{E65R} fused to a strong activation domain is not able to induce flowering in a *CONSTANS*-deficient mutant

A potential criticism of using NF-$YB2^{E65R}$ as a tool to demonstrate an NF-YA requirement in flowering is that we do not know if mutant NF-YB had altered interactions with other components involved in photoperiod-dependent flowering. In particular, we did not know if it might impact CO recruitment or binding to its CORE site. One approach to test this question might be a Y2H assay between NF-YB2 or NF-$YB2^{E65R}$ and CO. However, as we have previously published, we are unable to detect a direct interaction between NF-YB and CO or CO-Like proteins in Y2H assays [19]. We additionally attempted to replicate the Y3H experiments above, replacing NF-YA for CO, but did not observe yeast growth with either NF-YB2 or NF-$YB2^{E65R}$. However, we were concerned that this lack of a Y3H interaction could simply be a technical problem with the assay. Therefore, to further examine the potential impact of the NF-$YB2^{E65R}$ on CO function, we extended previous experiments by Tiwari *et al.* [49]. Here they showed that *NF-YB2* overexpression could not rescue the late flowering phenotype of a *CO* loss of function mutant (*co-9* allele); alternatively, when a strong transcriptional activation domain (called *EDLL*) was fused to *NF-YB2*, it was able to drive very early flowering in *co-9* plants. Therefore, we reasoned that if the *NF-$YB2^{E65R}$* flowering responses were due to loss of interaction with CO, *NF-$YB2^{E65R}$-EDLL* would be able to drive early flowering in a *co* mutant.

On the other hand, if NF-YA interactions were relevant in flowering, we should expect that an *NF-YB2*^{E65R}*-EDLL* construct would not be able to drive early flowering or rescue a *co* mutant.

We first examined overexpressed (35S) *NF-YB2-EDLL* in the same *co-9* loss of function allele. Consistent with Tiwari et al., we found that *co-9* plants transgenic for *p35S:NF-YB2-EDLL* flowered as early as 8–15 leaves (the *co-9* average was 40 leaves with no plants earlier than 30 leaves). However, *co-9* plants expressing either *p35S:NF-YB2* or *p35S:NF-YB2*^{E65R}*-EDLL* did not flower early and were indistinguishable from *co-9*. Here the impacts of the E65R mutation cannot be attributed to loss of interaction with CO. To further examine the E65R mutation, we additionally examined these constructs in Col-0 and the *co-2* mutant (Ler ecotype, Fig 5A and 5B): once again, while *NF-YB2* alone did not drive early flowering, *NF-YB2-EDLL* overexpressing plants were consistently earlier. However, in each case, *NF-YB2*^{E65R}*-EDLL* either caused later flowering (presumably the dominant negative effect suggested above, Fig 3A) or had no effect. We then used the *nf-yb2 nf-yb3* background where *NF-YB2* plants flowered at a mean of ~21 leaves and *NF-YB2-EDLL* flowered at ~12 leaves (Fig 5C); *NF-YB2*^{E65R}*-EDLL* was once again unable to alter flowering time. Short day grown plants, which mimic a *co* mutant because CO is unable to accumulate [2], told the same story—*NF-YB2-EDLL*, but not *NF-YB2*^{E65R}*-EDLL*, caused earlier flowering (Fig 5D). Finally, we repeated the entire transgenic panel in the loss of function *ft-10* mutant (Fig 5E). Importantly, all constructs, including *NF-YB2-EDLL*, failed to cause significantly earlier flowering in the *ft-10* genetic background. Collectively, this data adds additional evidence for NF-YA proteins as positive, *FT*-dependent regulators of photoperiod-dependent flowering.

NF-YA2-EDLL induces flowering in a CONSTANS-deficient mutant

We additionally hypothesized that if NF-YA2 is able to interact with NF-YB/NF-YC dimers on the *FT* promoter, attaching the *EDLL* domain to the *pNF-YA2:NF-YA2* construct would also induce flowering in *co* mutants. If true, this would significantly extend the NF-YB2^{E65R} and EMSA results above, ameliorating possible concerns about relying on NF-YB2^{E65R} as a proxy measure of NF-YA function. Again, we first tested flowering responses of 15–20 randomly selected T1 plants in the Col-0 background. As expected, both *pNF-YA2:NF-YA2* and *pNF-YA2:NF-YA2-EDLL* drove earlier flowering in Col-0 (Fig 6A). Alternatively, in the *co-2* background, *pNF-YA2:NF-YA2-EDLL* induced much earlier flowering (~20 leaves earlier than *co-2*), whereas the control *pNF-YA2:NF-YA2* did not (Fig 6B). As with *NF-YB2-EDLL* (Fig 5E), *NF-YA2-EDLL* was completely unable to induce flowering in the *ft-10* background (Fig 6C), indicating once again an *FT*-dependent, positive role for NF-YA proteins in flowering.

Discussion

Our initial understanding of NF-Y roles in flowering was primarily driven by evidence of physical interactions between individual NF-Y subunits and CO, as well as *in planta* overexpression analyses [15, 20]. Thereafter, loss of function mutations in HFD subunits identified specific *NF-YB* and *NF-YC* genes involved in flowering [18, 30, 50]. Demonstrating roles for *NF-YA*s has proven more difficult, since they appear to have redundant functions, mutants of a few members are lethal (including *NF-YA2*) and overexpressing them leads to substantially deleterious pleiotropic effects [37, 43, 44, 51, 52]. Here we have attempted to work around these difficulties with a variety of biochemical and genetic approaches. We provide a compelling body of evidence that NF-YA2 and NF-YA6, and perhaps other NF-YAs, can activate *FT* expression, and are *FT*-dependent, positive regulators of flowering.

Previously, NF-YAs were believed to act as negative regulators of flowering, because overexpression of two *NF-YA* genes, *NF-YA1* and *NF-YA4*, led to later flowering [20]. We noticed the

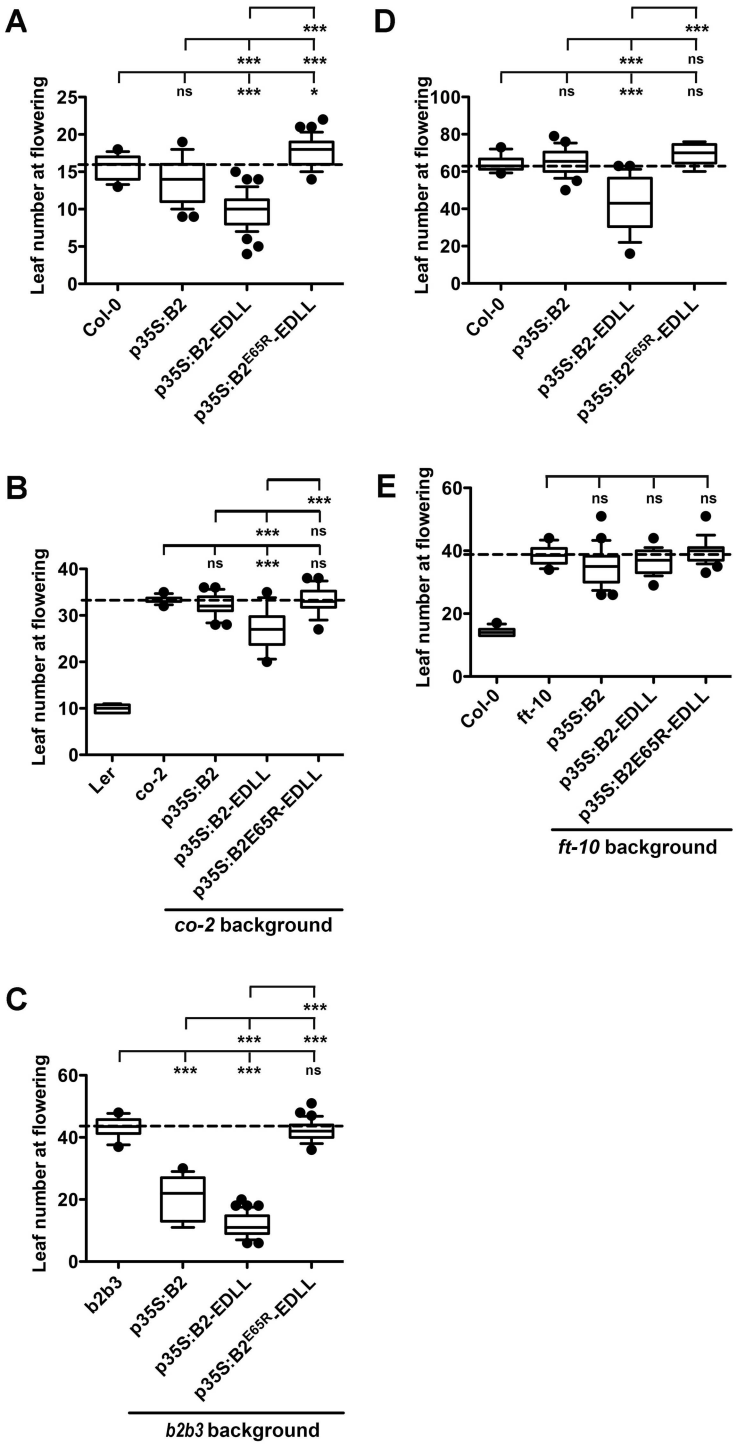

Fig 5. *NF-YB2-EDLL*, but not *NF-YB2^{E65R}-EDLL*, rescues late flowering in an *FT*-dependent manner. Flowering time quantification for 15–20 randomly selected T1 plants of *p35S*:*NF-YB2*, *p35S*:*NF-YB2-EDLL*, and *p35S*:*NF-YB2^{E65R}-EDLL* in A) Col-0 B) *co-2* C) *b2b3* D) short days E) *ft-10*. Asterisks represent significant differences derived from one-way ANOVA ($P < 0.05$) followed by Bonferroni's multiple comparison tests (*** $P < 0.001$; * $P < 0.05$).

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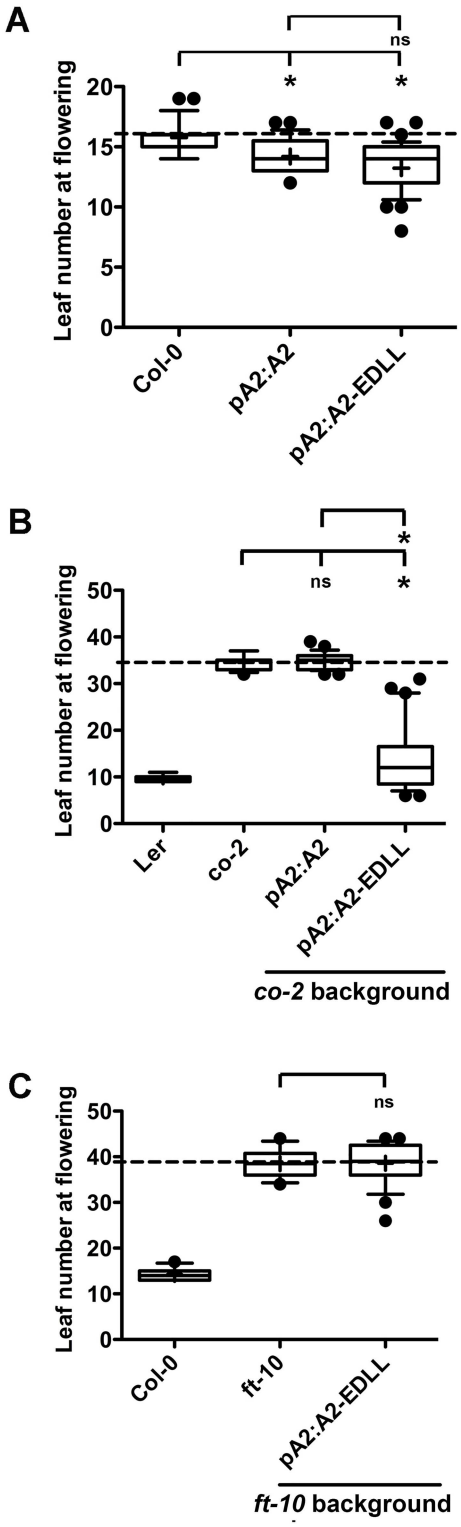

Fig 6. *pNF-YA2:NF-YA2-EDLL* can induce flowering in the absence of CO. Flowering time quantification for 15–20 randomly selected T1 plants in A) Col-0, B) *co-2*, and C) *ft-10*. Asterisks represent significant differences derived from one-way ANOVA ($P < 0.05$) followed by Bonferroni's multiple comparison tests.

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same response with *NF-YA7* and *NF-YA9* overexpressors. In another study by Leyva-Gonzalez [51], this was also the outcome of generalized overexpression of *NF-YA*s. NF-YA2 has also been demonstrated to be a negative regulator of a novel flowering response pathway, which is independent of the photoperiod dependent pathway, termed stress-mediated flowering responses [34]. Nevertheless, our observation of early flowering in *NF-YA2* overexpression lines is consistent with those recently reported [17]. Although, Hou et al. [17] suggested that NF-YA2 was a positive regulator of flowering time, it was suggested that this was mediated by interaction with a novel, non-*CCAAT cis* regulatory element called NFYBE in the *SOC1* promoter, and not the binding and regulation of *FT* expression. This seems at odds with existing evidence, as well as experiments presented here.

As reported for *co* mutants [40], multiple groups have demonstrated that *nf-yb* and *nf-yc* mutants also had strongly reduced *FT* expression and that these reductions were directly correlated with alterations in flowering time [18–20, 30, 53]. Likewise, overexpression of *NF-YB* and *NF-YC* genes was associated with *FT* upregulation [18, 30, 49, 54, 55]. Mutations in *cis*-regulatory elements bound by either CO or NF-Y complexes in the *FT* promoter (*CCAAT* and/or CORE, respectively) also reduced *FT* expression in a manner that was directly correlated with the severity of flowering delays [16, 18, 19, 32]. Further, constitutive overexpression of *CO* drove increased *FT* expression and early flowering, but these phenotypes were strongly reduced or eliminated in *nf-yb* and *nf-yc* mutants or when the -5.3kb *CCAAT* site was eliminated [19, 32, 49]. Finally, multiple labs have shown *in vivo* and *in vitro* binding of NF-Y and CO proteins to the *FT* promoter and mutations in the associated *CCAAT* and CORE regulatory elements additively reduced *FT* expression and delayed flowering [7, 8, 16, 32]. Thus, it remains very well-supported that photoperiod-dependent flowering is mediated through direct regulation of *FT* by CO and NF-Y complexes.

Elegant genetic experiments previously demonstrated that *SOC1* activation is downstream of *FT* [42]. Therefore, if NF-Ys are directly binding and activating *SOC1* to activate photoperiod-dependent flowering, *FT* loss of function alleles (such as *ft-10* used here) should not have impaired this function. However, we find that *p35S:NF-YA-EDLL* and *p35S:NF-YB-EDLL* cannot drive early flowering in the absence of *FT*, strongly suggesting that *SOC1* is not their only target in photoperiod-dependent flowering. We do not rule out the possibility that the NF-Y are also involved in the direct regulation of *SOC1* and this may be an important element of the gibberellic acid flowering pathway [56]. However, regulation of *SOC1* alone cannot readily explain the flowering phenotypes discussed here.

Regulation of the *FT* promoter is influenced by a plethora of pathways and numerous *cis*-regulatory elements continue to emerge [16, 32, 33, 57]. One of these is the -5.3kb *CCAAT* enhancer site, where both deletions and mutations significantly delay flowering time [16, 32, 58]. We provide here formal *in vitro* evidence that complexes formed by NF-YA2 and NF-YA6, associated with NF-YB2/NF-YC3, robustly and specifically bind to this site. Interestingly, the phenotype of the -5.3kb *CCAAT* mutant was not as strong as those from *nf-y* HFD loss of function alleles [16, 18], implying that there must be additional *CCAAT* sites bound by the NF-Y trimer in the *FT* promoter or that NF-Y subunits also regulate non-*CCAAT* sites. Another set of important sites responsible for CO activation, CORE, are in the proximal promoter [8, 32]. Indeed, the near complete loss of photoperiod-dependent flowering responses in *nf-yb2 nf-yb3* and *co* mutants strongly argues that NF-Y complexes and CO must be necessary for function at both *cis*-regulatory regions. In keeping with this, we recently showed that NF-Y, bound to the -5.3kb *CCAAT*, and CO, bound to CORE sites, physically interact via a chromatin loop. Further, simultaneous mutations in the -5.3kb *CCAAT*, CORE1 and CORE2 sites in the *FT* promoter nearly eliminated rescue of an *ft-10* mutant [16]. The importance of the -5.3 kb *CCAAT* element implies a role of the sequence-specific subunit NF-YA; however,

the interactions of the HFD subunits with CO, and the resulting enhancer-promoter connections through CORE, made the direct demonstration of NF-YA function in *FT* expression and flowering all the more important.

NF-YB2^{E65R} overexpressors were not able to rescue the late flowering phenotype of the *nf-yb2 nf-yb3* mutant. We formally excluded that this was due to expression levels and we could also exclude that the mutant folded incorrectly for two reasons: 1) Recombinant production in *E. coli* recovered WT and E65R as soluble proteins when co-expressed with NF-YC3, and indeed both were easily purified, and 2) The mutant had a dominant negative effect on flowering time when overexpressed in Col-0 plants. A similar conclusion on the dominant negative nature of the glutamic acid mutation was made for rat NF-YB (CBF-A) *in vitro* [22], but this is the first demonstration that it could also act *in vivo*. The likeliest explanation for the dominant negative behavior of NF-YB2^{E65R} is related to formation of HFD heterodimers impaired in trimer formation, and hence normal NF-Y function—i.e., it is possible that they subtract functional NF-YCs, which would otherwise enter the normal trimerization/*CCAAT*-binding processes. Obviously, we cannot formally rule out the possibility that the NF-YB2^{E65R} mutant lost interaction with proteins other than NF-YA and that this resulted in the lack of rescue of late flowering.

To rule out the possibility that the NF-YB2^{E65R} flowering phenotypes were possibly due to loss of interaction with CO, we used the EDLL transactivation domain. CO was previously demonstrated to provide an activation domain for the NF-Y complex and NF-YB2 was able to drive flowering in the absence of CO when fused to the EDLL activation domain [49]. However, in the current study, *p35S:NF-YB2^{E65R}-EDLL* was not able to induce flowering, indicating that while CO provides an activation domain for the NF-Y complex, the HFD dimer is non-functional in the absence of NF-YA. Our EMSA data further connects an NF-YA requirement to the capacity to bind at *CCAAT* elements. Finally, the flowering phenotypes for *pNF-YA2:NF-YA2-EDLL* were essentially the same as *p35S:NF-YB2-EDLL*. Both constructs were able to induce flowering in *co* mutants, were not able to induce flowering in *ft-10* mutants, and drove earlier flowering in Col-0.

Collectively, these data strongly suggest that NF-YA proteins regulate photoperiod-dependent flowering by directly binding and modifying the expression of *FT*.

Methods

Multiple sequence alignments. Protein sequences were obtained from TAIR (http://www.arabidopsis.org [59] or National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and manipulated in TextWrangler (http://www.barebones.com) Multiple sequence alignments were made using ClustalX [60] and shaded within Geneious (http://www.geneious.com/).

Generation of overexpression constructs. The *p35S:NF-YB2* and the ten *p35S:NF-YA* constructs were previously described [37, 47], as was the *35S* promoter [61]. *NF-YB2^{E65R}* was amplified from cDNA using mutagenic PCR. *pNF-YA2:NF-YA2* was amplified using genomic DNA with the promoter region starting approximately 1 KB upstream of the start codon. The proof reading enzyme Pfu Ultra II (cat#600670; Agilent Technologies) was used for PCR reactions and the resulting fragments were ligated into GATEWAY™ entry vector pENTR/D-TOPO (cat#45–0218; Invitrogen). The EDLL domain [49] was amplified from cDNA and contained *Acs1* sites, which were used to clone the EDLL domain into the pENTR/D-TOPO backbone of *NF-YB2*, *NF-YA2* and *NF-YB2^{E65R}* entry clones. All entry clones generated were sequenced and other than the point mutation were identical to sequences at TAIR (http://www.arabidopsis.org [59]. Entry clones were sub-cloned into the following destination vectors

using the GATEWAY™ LR Clonease II reaction kit (cat#56485; Invitrogen): *NF-YB2*^{E65R}, *NF-YB2-EDLL* and *NF-YB2*^{E65R}*-EDLL* into pEarlyGate101 (YFP-HA tag) for selection using BASTA resistance [62] and pK7FWG2 (GFP tag) for selection using kanamycin resistance [63]; *pNF-YA2*:*NF-YA2* and *pNF-YA2*:*NF-YA2-EDLL* into pEarlyGate301 (HA tag) [62]. S1 Table lists primer sequences used for cloning and mutagenesis.

Plant transformation, cultivation and flowering time experiments. *Arabidopsis thaliana* ecotype Columbia (Col-0) was the wild type for all experiments, except the *co-2* allele in the Ler ecotype. *nf-yb2 nf-yb3*, *ft-10*, *co-2*, and *co-9* [42, 47, 64, 65] were previously described. Plants were transformed using Agrobacterium mediated floral dipping [66]. Plants were cultivated in a custom-built walk-in chamber under standard long day conditions (16h light/8h dark) or short day conditions (8h light/ 16h dark) using plant growth conditions previously described [37]. All transgenic plants were selected by spraying with the herbicide glufosinate-ammonium (BASTA), except in the *co-9* background where selection was done on 50μg/ml kanamycin plates. Leaf number at flowering was measured as the total number of rosette and cauline leaves on the primary axis at flowering.

Protein expression and purification. The cDNAs encoding for NF-YA2 (aa 134–207) and NF-YA6 (aa 170–237) were obtained by gene synthesis (Eurofins Genomics) and cloned into pnEA/tH [67] by restriction ligation with NdeI and BamHI to obtain C-terminal 6His-tag fusions. The CCT domain of CO (aa 290–352), with the addition of a 5' ATG, was cloned into pnEA/tH via PCR amplification followed by restriction ligation with XhoI and MunI to obtain C-terminal 6His-tag fusions. Clones were verified by sequence analysis. *NF-YB2* mutant cDNA, encoding for aa 24–116 with residue E65 mutated to R (*NF-YB2*^{E65R}) was obtained by gene synthesis and subcloned in pET15b to obtain N-terminal 6His-tag fusion. 6His-NF-YB2 or 6His-NF-YB2^{E65R}/NF-YC3 soluble HFD dimers were produced by co-expression in *E. coli* BL21(DE3) and purified by ion metal affinity chromatography (IMAC) as described in [68]. NF-YA2-6His, NF-YA6-6His or CO-6His were expressed in BL21(DE3) by IPTG induction (0.4mM IPTG for 4h at 25C) and purified by IMAC (HisSelect, SIGMA-Aldrich) in buffer A (10mM Tris pH 8.0, 400mM NaCl, 2mM $MgCl_2$, 5mM imidazole). Purified proteins were eluted in Buffer A containing 100mM imidazole, and dialysed against Buffer B (10mM Tris-Cl pH 8.0, 400mM NaCl, 2mM DTT, 10% glycerol).

Electrophoretic Mobility Shift Assays. EMSA analyses were performed essentially as previously described [16, 67, 68]. Heterotrimer formation and CCAAT-box DNA-binding of WT or mutant NF-YB2/NF-YC3 dimers was assessed by addition of purified NF-YAs (or CO) using the Cy5-labeled *FT CCAAT* probe [16]. DNA binding reactions (16μl) (20nM *FT CCAAT* probe, 12mM Tris-HCl pH 8.0, 50mM KCl, 62.5mM NaCl, 0.5mM EDTA, 5mM $MgCl_2$, 2.5mM DTT, 0.2 mg/ml BSA, 5% glycerol, 6.25ng/μl poly dA-dT) were incubated with WT or mutant NF-YB2/NF-YC3 dimers (60nM), with or without NF-YA2 or -YA6 (or CO), as indicated in Fig 4. Proteins were pre-mixed in Buffer B containing 0.1 mg/ml BSA, then added to DNA binding mixes. After 30min incubation at 30C, binding reactions were loaded on 6% polyacrylamide gels and separated by electrophoresis in 0.25X TBE. Fluorescence gel images were obtained and analyzed with a Chemidoc™ MP system and ImageLab™ software (Bio-Rad).

Western blot. Total protein was extracted by grinding in lysis buffer (20mM Tris, pH 8.0, 150mM NaCl, 1mM EDTA, pH 8.0, 1% Triton X-100, 1% SDS with fresh 5mM DTT, 10mM protease inhibitor). NF-YB2-YFP/HA and NF-YB2^{E65R}-YFP/HA were detected using high affinity anti-HA primary antibody (cat#11 867 423 001; Roche) and goat anti-rat secondary antibody (cat#SC-2032; Santa Cruz Biotechnology). Horseradish peroxidase-based ECL plus reagent was used for visualization in a Bio-Rad ChemiDoc XRS imaging system. The membrane was stained with Ponceau S (cat#P3504; Sigma-Aldrich) to determine equivalent loading and transfer efficiency.

Confocal imaging. *p35S:NF-YB2-YFP* and *p35S:NF-YB2^{E65R}:YFP* in *nf-yb2 nf-yb3* background, and *nf-yb2 nf-yb3* seeds were cold stratified in the dark for 48-h then germinated and grown on B5 media under 24hr light. Six to seven-day-old seedlings were counterstained with propidium iodide (PI) (50μg/mL) for five minutes, washed in DI water for five minutes and whole mounted in fresh DI water on standard slides. Hypocotyls were imaged with an Olympus FluoView 500 using a 60X WLSM objective. XYZ scans were taken with line sequential scanning mode where fluorescent signals were sampled using a filter-based detection system optimized for YFP and PI with chloroplast autofluorescence also detected in the latter. YFP was excited using a 488nm Argon laser whereas PI was excited using a 543nm Helium Neon laser. Approximately 50 serial sections were imaged with a cubic voxel size of 414nm x 414nm x 414nm. Image processing took place in ImageJ (http://rsb.info.nih.gov/ij/) where average intensity projections were taken from YFP and PI channels and merged.

Yeast two-hybrid (Y2H) and three-hybrid (Y3H) analysis. Entry clones of *NF-YA2* and *NF-YC9*, which were previously described [19, 37], were subcloned into pDEST™22 (Invitrogen) and pTFT1 [69] respectively to obtain an activation domain (AD) and bridge construct. The DNA binding domain (DBD) and AD constructs for *NF-YB2*, *CO* and *NF-YC9* were previously described [19]. The plasmids were transferred to the yeast strains MaV203 (Invitrogen) for Y2H and PJ69-4α [70] for Y3H analysis. Protein interactions were tested according to the ProQuest™ manual (Invitrogen). For the X-Gal assay nitrocellulose membranes were frozen in liquid nitrogen and placed on a filter paper saturated with Z-buffer containing X-Gal (5-bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside, Gold Biotechnology, cat#Z4281L). For the synthetic dropout medium lacking the amino acid Histidine, 5mM 3-amino-1,2,4-triazole (3-AT) was added to eliminate nonspecific activation. All experiments were repeated with identical results.

qPCR analysis. Total RNA was collected from seven-day-old or nine-day-old seedlings according to instructions in the E.Z.N.A Plant RNA Kit (cat#R6827-01; Omega Biotek). For each genotype, we analyzed three or four independent biological replicates. First-strand cDNA synthesis was performed as previously described [37]. For qPCR a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) with the SYBR Green qPCR Master Mix (cat#K0222; Fermentas) was used. Results were analyzed using CFX Manager™ (Bio-Rad) where samples were normalized to a constitutively expressed reference gene At2G32170 [71]. S1 Table lists primer sequences used for qPCR analysis.

Supporting Information

S1 Table. List of Primers.
(DOCX)

S1 Fig. *NF-YA2* is expressed in *pNF-YA2:NF-YA2* plants. Quantification of *NF-YA2* expression in *pNF-YA2:NF-YA2-1* plants used for qPCR analysis. Asterisks represent significant differences derived from student's T-test ($P < 0.05$).
(PDF)

S2 Fig. NF-YB2^{E65R} is expressed in the *nf-yb2 nf-yb3* background. (**A**) Confocal images of NF-YB2 and NF-YB2^{E65R} protein localization in stable plant lines. (**B**) Protein expression in 12 individual T1 *p35S:NF-YB2^{E65R}* plants compared to a stable strongly expressed *p35S:NF-YB2* in the *nf-yb2 nf-yb3* background. Regardless of the level of expression, *p35S:NF-YB2^{E65R}* (flowering at > 31 leaves) was not able to rescue the *nf-yb2 nf-yb3* late flowering phenotype, whereas *p35S:NF-YB2* was readily able to rescue the late flowering phenotype (flowering at 12 leaves). (C) Protein expression in 12 individual T1 *p35S:NF-YB2^{E65R}* plants with

individual flowering times shown. (**D**) Protein expression in two stable plant lines each for *p35S:NF-YB2* and *p35S:NF-YB2*^{E65R} in the *nf-yb2 nf-yb3* background. Note here that all four stable lines had the same approximate level of protein expression, however the *p35S:NF-YB2* lines were able to rescue the *nf-yb2 nf-yb3* late flowering phenotype, whereas *p35S:NF-YB2*^{E65R} were not able to rescue *nf-yb2 nf-yb3*.
(PDF)

S3 Fig. *FT CCAAT* is bound by the NF-Y trimer and not by the CO/NF-YB2/NF-YC3 trimer. EMSA assays were performed using the fluorescently labeled *FT CCAAT* DNA probe (20 nM). DNA binding was assayed by addition of CO (lanes 2–5) or NF-YAs, as indicated (lanes 6–9; 10–13), at increasing protein concentration (60, 120, 180, or 240 nM) in the presence of the WT NF-YB2/NF-YC3 HFD dimer (WT, 40 nM). As a control, the probe was incubated with the HFD dimer alone (40 nM, lane 1), or with the NF-YA2 protein (YA2, 240 nM, lane 14). The NF-Y/DNA complex is indicated by a labeled arrowhead. fp: free probe.
(PDF)

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References

1. Song YH, Shim JS, Kinmonth-Schultz HA, Imaizumi T. Photoperiodic flowering: time measurement mechanisms in leaves. Annu Rev Plant Biol. 2015; 66:441–64. doi: 10.1146/annurev-arplant-043014-115555 PMID: 25534513
2. Suarez-Lopez P, Wheatley K, Robson F, Onouchi H, Valverde F, Coupland G. CONSTANS mediates between the circadian clock and the control of flowering in Arabidopsis. Nature. 2001; 410(6832):1116–20. doi: 10.1038/35074138 PMID: 11323677
3. Jang S, Marchal V, Panigrahi KC, Wenkel S, Soppe W, Deng XW, et al. Arabidopsis COP1 shapes the temporal pattern of CO accumulation conferring a photoperiodic flowering response. Embo J. 2008; 27 (8):1277–88. doi: 10.1038/emboj.2008.68 PMID: 18388858
4. Liu LJ, Zhang YC, Li QH, Sang Y, Mao J, Lian HL, et al. COP1-mediated ubiquitination of CONSTANS is implicated in cryptochrome regulation of flowering in Arabidopsis. Plant Cell. 2008; 20(2):292–306. doi: 10.1105/tpc.107.057281 PMID: 18296627
5. Valverde F, Mouradov A, Soppe W, Ravenscroft D, Samach A, Coupland G. Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. Science. 2004; 303(5660):1003–6. doi: 10.1126/science.1091761 PMID: 14963328
6. Hayama R, Coupland G. The molecular basis of diversity in the photoperiodic flowering responses of Arabidopsis and rice. Plant Physiol. 2004; 135(2):677–84. doi: 10.1104/pp.104.042614 PMID: 15208414
7. Song YH, Smith RW, To BJ, Millar AJ, Imaizumi T. FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science. 2012; 336(6084):1045–9. Epub 2012/05/26. doi: 10.1126/science.1219644 PMID: 22628657
8. Tiwari SB, Shen Y, Chang HC, Hou Y, Harris A, Ma SF, et al. The flowering time regulator CONSTANS is recruited to the FLOWERING LOCUS T promoter via a unique cis-element. New Phytol. 2010; 187 (1):57–66. Epub 2010/04/22. doi: 10.1111/j.1469-8137.2010.03251.x PMID: 20406410

9. Lin MK, Belanger H, Lee YJ, Varkonyi-Gasic E, Taoka K, Miura E, et al. FLOWERING LOCUS T protein may act as the long-distance florigenic signal in the cucurbits. Plant Cell. 2007; 19(5):1488–506. doi: 10.1105/tpc.107.051920 PMID: 17540715
10. Corbesier L, Vincent C, Jang S, Fornara F, Fan Q, Searle I, et al. FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. Science. 2007; 316(5827):1030–3. doi: 10.1126/science.1141752 PMID: 17446353
11. Mathieu J, Warthmann N, Kuttner F, Schmid M. Export of FT protein from phloem companion cells is sufficient for floral induction in Arabidopsis. Current biology: CB. 2007; 17(12):1055–60. doi: 10.1016/j.cub.2007.05.009 PMID: 17540570
12. Tamaki S, Matsuo S, Wong HL, Yokoi S, Shimamoto K. Hd3a protein is a mobile flowering signal in rice. Science. 2007; 316(5827):1033–6. doi: 10.1126/science.1141753 PMID: 17446351
13. Pose D, Yant L, Schmid M. The end of innocence: flowering networks explode in complexity. Curr Opin Plant Biol. 2012; 15(1):45–50. doi: 10.1016/j.pbi.2011.09.002 PMID: 21974961
14. Andres F, Coupland G. The genetic basis of flowering responses to seasonal cues. Nat Rev Genet. 2012; 13(9):627–39. doi: 10.1038/nrg3291 PMID: 22898651
15. Ben-Naim O, Eshed R, Parnis A, Teper-Bamnolker P, Shalit A, Coupland G, et al. The CCAAT binding factor can mediate interactions between CONSTANS-like proteins and DNA. Plant J. 2006; 46(3):462–76. doi: 10.1111/j.1365-313X.2006.02706.x PMID: 16623906
16. Cao S, Kumimoto RW, Gnesutta N, Calogero AM, Mantovani R, Holt BF III. A Distal CCAAT/NUCLEAR FACTOR Y Complex Promotes Chromatin Looping at the FLOWERING LOCUS T Promoter and Regulates the Timing of Flowering in Arabidopsis. Plant Cell. 2014; 26:1009–17. doi: 10.1105/tpc.113.120352 PMID: 24610724
17. Hou X, Zhou J, Liu C, Liu L, Shen L, Yu H. Nuclear factor Y-mediated H3K27me3 demethylation of the SOC1 locus orchestrates flowering responses of Arabidopsis. Nature communications. 2014; 5:4601. doi: 10.1038/ncomms5601 PMID: 25105952
18. Kumimoto RW, Adam L, Hymus GJ, Repetti PP, Reuber TL, Marion CM, et al. The Nuclear Factor Y subunits NF-YB2 and NF-YB3 play additive roles in the promotion of flowering by inductive long-day photoperiods in Arabidopsis. Planta. 2008; 228(5):709–23. Epub 2008/07/05. doi: 10.1007/s00425-008-0773-6 PMID: 18600346
19. Kumimoto RW, Zhang Y, Siefers N, Holt BF III. NF-YC3, NF-YC4 and NF-YC9 are required for CONSTANS-mediated, photoperiod-dependent flowering in Arabidopsis thaliana. Plant J. 2010; 63:379–91. Epub 2010/05/22. doi: 10.1111/j.1365-313X.2010.04247.x PMID: 20487380
20. Wenkel S, Turck F, Singer K, Gissot L, Le Gourrierec J, Samach A, et al. CONSTANS and the CCAAT box binding complex share a functionally important domain and interact to regulate flowering of Arabidopsis. Plant Cell. 2006; 18(11):2971–84. doi: 10.1105/tpc.106.043299 PMID: 17138697
21. Sinha S, Maity SN, Lu J, de Crombrugghe B. Recombinant rat CBF-C, the third subunit of CBF/NFY, allows formation of a protein-DNA complex with CBF-A and CBF-B and with yeast HAP2 and HAP3. Proc Natl Acad Sci U S A. 1995; 92(5):1624–8. PMID: 7878029
22. Sinha S, Kim IS, Sohn KY, de Crombrugghe B, Maity SN. Three classes of mutations in the A subunit of the CCAAT-binding factor CBF delineate functional domains involved in the three-step assembly of the CBF-DNA complex. Mol Cell Biol. 1996; 16(1):328–37. PMID: 8524312
23. Kahle J, Baake M, Doenecke D, Albig W. Subunits of the heterotrimeric transcription factor NF-Y are imported into the nucleus by distinct pathways involving importin beta and importin 13. Mol Cell Biol. 2005; 25(13):5339–54. doi: 10.1128/MCB.25.13.5339-5354.2005 PMID: 15964792
24. Frontini M, Imbriano C, Manni I, Mantovani R. Cell cycle regulation of NF-YC nuclear localization. Cell Cycle. 2004; 3(2):217–22. PMID: 14712092
25. Mantovani R. The molecular biology of the CCAAT-binding factor NF-Y. Gene. 1999; 239(1):15–27. PMID: 10571030
26. Kusnetsov V, Landsberger M, Meurer J, Oelmuller R. The assembly of the CAAT-box binding complex at a photosynthesis gene promoter is regulated by light, cytokinin, and the stage of the plastids. J Biol Chem. 1999; 274(50):36009–14. PMID: 10585491
27. Nardini M, Gnesutta N, Donati G, Gatta R, Forni C, Fossati A, et al. Sequence-specific transcription factor NF-Y displays histone-like DNA binding and H2B-like ubiquitination. Cell. 2013; 152(1–2):132–43. Epub 2013/01/22. doi: 10.1016/j.cell.2012.11.047 PMID: 23332751
28. Laloum T, Baudin M, Frances L, Lepage A, Billault-Penneteau B, Cerri MR, et al. Two CCAAT-box-binding transcription factors redundantly regulate early steps of the legume-rhizobia endosymbiosis. Plant J. 2014; 79(5):757–68. doi: 10.1111/tpj.12587 PMID: 24930743

29. Petroni K, Kumimoto RW, Gnesutta N, Calvenzani V, Fornari M, Tonelli C, et al. The Promiscuous Life of Plant NUCLEAR FACTOR Y Transcription Factors. Plant Cell. 2012; 24(12):4777–92. Epub 2013/01/01. doi: 10.1105/tpc.112.105734 PMID: 23275578

30. Cai X, Ballif J, Endo S, Davis E, Liang M, Chen D, et al. A putative CCAAT-binding transcription factor is a regulator of flowering timing in Arabidopsis. Plant Physiol. 2007; 145(1):98–105. doi: 10.1104/pp.107.102079 PMID: 17631525

31. Chen NZ, Zhang XQ, Wei PC, Chen QJ, Ren F, Chen J, et al. AtHAP3b plays a crucial role in the regulation of flowering time in Arabidopsis during osmotic stress. Journal of biochemistry and molecular biology. 2007; 40(6):1083–9. PMID: 18047807

32. Adrian J, Farrona S, Reimer JJ, Albani MC, Coupland G, Turck F. cis-Regulatory elements and chromatin state coordinately control temporal and spatial expression of FLOWERING LOCUS T in Arabidopsis. Plant Cell. 2010; 22(5):1425–40. Epub 2010/05/18. doi: 10.1105/tpc.110.074682 PMID: 20472817

33. Hu K, Xu C, Ni H, Xu Z, Wang Y, Xu S, et al. Mir-218 contributes to the transformation of 5-Aza/GF induced umbilical cord mesenchymal stem cells into hematopoietic cells through the MITF pathway. Mol Biol Rep. 2014; 41(7):4803–16. doi: 10.1007/s11033-014-3351-y PMID: 24696000

34. Xu MY, Zhang L, Li WW, Hu XL, Wang MB, Fan YL, et al. Stress-induced early flowering is mediated by miR169 in Arabidopsis thaliana. J Exp Bot. 2014; 65(1):89–101. doi: 10.1093/jxb/ert353 PMID: 24336445

35. Siefers N, Dang KK, Kumimoto RW, Bynum WE IV, Tayrose G, Holt BF III. Tissue-specific expression patterns of Arabidopsis NF-Y transcription factors suggest potential for extensive combinatorial complexity. Plant Physiol. 2009; 149(2):625–41. doi: 10.1104/pp.108.130591 PMID: 19019982

36. Xing Y, Fikes JD, Guarente L. Mutations in yeast HAP2/HAP3 define a hybrid CCAAT box binding domain. Embo J. 1993; 12(12):4647–55. PMID: 8223474

37. Siriwardana CL, Kumimoto RW, Jones DS, Holt BF 3rd. Gene Family Analysis of the Transcription Factors Reveals Opposing Abscisic Acid Responses During Seed Germination. Plant Mol Biol Report. 2014; 32(5):971–86. doi: 10.1007/s11105-014-0704-6 PMID: 25190903

38. An H, Roussot C, Suarez-Lopez P, Corbesier L, Vincent C, Pineiro M, et al. CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of Arabidopsis. Development. 2004; 131(15):3615–26. doi: 10.1242/dev.01231 PMID: 15229176

39. Notaguchi M, Abe M, Kimura T, Daimon Y, Kobayashi T, Yamaguchi A, et al. Long-distance, graft-transmissible action of Arabidopsis FLOWERING LOCUS T protein to promote flowering. Plant Cell Physiol. 2008; 49(11):1645–58. Epub 2008/10/14. doi: 10.1093/pcp/pcn154 PMID: 18849573

40. Kobayashi Y, Kaya H, Goto K, Iwabuchi M, Araki T. A pair of related genes with antagonistic roles in mediating flowering signals. Science. 1999; 286(5446):1960–2. Epub 1999/12/03. PMID: 10583960

41. Kardailsky I, Shukla VK, Ahn JH, Dagenais N, Christensen SK, Nguyen JT, et al. Activation tagging of the floral inducer FT. Science. 1999; 286(5446):1962–5. PMID: 10583961

42. Yoo SK, Chung KS, Kim J, Lee JH, Hong SM, Yoo SJ, et al. CONSTANS activates SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 through FLOWERING LOCUS T to promote flowering in Arabidopsis. Plant Physiol. 2005; 139(2):770–8. doi: 10.1104/pp.105.066928 PMID: 16183837

43. Pagnussat GC, Yu HJ, Ngo QA, Rajani S, Mayalagu S, Johnson CS, et al. Genetic and molecular identification of genes required for female gametophyte development and function in Arabidopsis. Development. 2005; 132(3):603–14. Epub 2005/01/07. doi: 10.1242/dev.01595 PMID: 15634699

44. Meinke D, Muralla R, Sweeney C, Dickerman A. Identifying essential genes in Arabidopsis thaliana. Trends in plant science. 2008; 13(9):483–91. Epub 2008/08/08. doi: 10.1016/j.tplants.2008.06.003 PMID: 18684657

45. Stephenson TJ, McIntyre CL, Collet C, Xue GP. Genome-wide identification and expression analysis of the NF-Y family of transcription factors in Triticum aestivum. Plant Mol Biol. 2007; 65(1–2):77–92. doi: 10.1007/s11103-007-9200-9 PMID: 17598077

46. Thirumurugan T, Ito Y, Kubo T, Serizawa A, Kurata N. Identification, characterization and interaction of HAP family genes in rice. Mol Genet Genomics. 2008; 279(3):279–89. Epub 2008/01/15. doi: 10.1007/s00438-007-0312-3 PMID: 18193457

47. Cao S, Kumimoto RW, Siriwardana CL, Risinger JR, Holt BF 3rd. Identification and characterization of NF-Y transcription factor families in the monocot model plant Brachypodium distachyon. PLoS One. 2011; 6(6):e21805. Epub 2011/07/09. doi: 10.1371/journal.pone.0021805 PMID: 21738795

48. Kim IS, Sinha S, de Crombrugghe B, Maity SN. Determination of functional domains in the C subunit of the CCAAT-binding factor (CBF) necessary for formation of a CBF-DNA complex: CBF-B interacts simultaneously with both the CBF-A and CBF-C subunits to form a heterotrimeric CBF molecule. Mol Cell Biol. 1996; 16(8):4003–13. Epub 1996/08/01. PMID: 8754798

49. Tiwari SB, Belachew A, Ma SF, Young M, Ade J, Shen Y, et al. The EDLL motif: a potent plant transcriptional activation domain from AP2/ERF transcription factors. Plant J. 2012; 70(5):855–65. Epub 2012/02/11. doi: 10.1111/j.1365-313X.2012.04935.x PMID: 22321262

50. Kumimoto RW, Siriwardana CL, Gayler KK, Risinger JR, Siefers N, Holt BF 3rd. NUCLEAR FACTOR Y Transcription Factors Have Both Opposing and Additive Roles in ABA-Mediated Seed Germination. PLoS One. 2013; 8(3):e59481. Epub 2013/03/26. doi: 10.1371/journal.pone.0059481 PMID: 23527203

51. Leyva-Gonzalez MA, Ibarra-Laclette E, Cruz-Ramirez A, Herrera-Estrella L. Functional and transcriptome analysis reveals an acclimatization strategy for abiotic stress tolerance mediated by Arabidopsis NF-YA family members. PLoS One. 2012; 7(10):e48138. Epub 2012/11/03. doi: 10.1371/journal.pone.0048138 PMID: 23118940

52. Mu J, Tan H, Hong S, Liang Y, Zuo J. Arabidopsis Transcription Factor Genes NF-YA1, 5, 6, and 9 Play Redundant Roles in Male Gametogenesis, Embryogenesis, and Seed Development. Molecular plant. 2012. Epub 2012/08/31.

53. Yan WH, Wang P, Chen HX, Zhou HJ, Li QP, Wang CR, et al. A major QTL, Ghd8, plays pleiotropic roles in regulating grain productivity, plant height, and heading date in rice. Molecular plant. 2011; 4 (2):319–30. Epub 2010/12/15. doi: 10.1093/mp/ssq070 PMID: 21148627

54. Hackenberg D, Wu Y, Voigt A, Adams R, Schramm P, Grimm B. Studies on differential nuclear translocation mechanism and assembly of the three subunits of the Arabidopsis thaliana transcription factor NF-Y. Molecular plant. 2012; 5(4):876–88. Epub 2011/12/27. doi: 10.1093/mp/ssr107 PMID: 22199235

55. Liang M, Hole D, Wu J, Blake T, Wu Y. Expression and functional analysis of NUCLEAR FACTOR-Y, subunit B genes in barley. Planta. 2012; 235(4):779–91. doi: 10.1007/s00425-011-1539-0 PMID: 22042327

56. Xu F, Li T, Xu PB, Li L, Du SS, Lian HL, et al. DELLA proteins physically interact with CONSTANS to regulate flowering under long days in Arabidopsis. FEBS letters. 2016; 590(4):541–9. doi: 10.1002/1873-3468.12076 PMID: 26801684

57. Pin PA, Nilsson O. The multifaceted roles of FLOWERING LOCUS T in plant development. Plant Cell Environ. 2012; 35(10):1742–55. Epub 2012/06/16. doi: 10.1111/j.1365-3040.2012.02558.x PMID: 22697796

58. Liu L, Adrian J, Pankin A, Hu J, Dong X, von Korff M, et al. Induced and natural variation of promoter length modulates the photoperiodic response of FLOWERING LOCUS T. Nature communications. 2014; 5:4558. doi: 10.1038/ncomms5558 PMID: 25087553

59. Huala E, Dickerman AW, Garcia-Hernandez M, Weems D, Reiser L, LaFond F, et al. The Arabidopsis Information Resource (TAIR): a comprehensive database and web-based information retrieval, analysis, and visualization system for a model plant. Nucleic Acids Res. 2001; 29(1):102–5. Epub 2000/01/11. PMID: 11125061

60. Thompson JD, Gibson TJ, Higgins DG. Multiple sequence alignment using ClustalW and ClustalX. Curr Protoc Bioinformatics. 2002; Chapter 2: Unit 2 3.

61. Kay R, Chan A, Daly M, McPherson J. Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science. 1987; 236:1299–302. doi: 10.1126/science.236.4806.1299 PMID: 17770331

62. Earley KW, Haag JR, Pontes O, Opper K, Juehne T, Song K, et al. Gateway-compatible vectors for plant functional genomics and proteomics. Plant J. 2006; 45(4):616–29. doi: 10.1111/j.1365-313X.2005.02617.x PMID: 16441352

63. Karimi M, Inze D, Depicker A. GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends in plant science. 2002; 7(5):193–5. PMID: 11992820

64. Koornneef M, Hanhart CJ, van der Veen JH. A genetic and physiological analysis of late flowering mutants in Arabidopsis thaliana. Molecular & general genetics: MGG. 1991; 229(1):57–66.

65. Balasubramanian S, Sureshkumar S, Lempe J, Weigel D. Potent induction of Arabidopsis thaliana flowering by elevated growth temperature. PLoS genetics. 2006; 2(7):e106. doi: 10.1371/journal.pgen.0020106 PMID: 16839183

66. Clough SJ, Bent AF. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 1998; 16(6):735–43. Epub 1999/03/09. PMID: 10069079

67. Diebold ML, Fribourg S, Koch M, Metzger T, Romier C. Deciphering correct strategies for multiprotein complex assembly by co-expression: application to complexes as large as the histone octamer. J Struct Biol. 2011; 175(2):178–88. doi: 10.1016/j.jsb.2011.02.001 PMID: 21320604

68. Calvenzani V, Testoni B, Gusmaroli G, Lorenzo M, Gnesutta N, Petroni K, et al. Interactions and CCAAT-Binding of Arabidopsis thaliana NF-Y Subunits. PLoS One. 2012; 7(8):e42902. Epub 2012/08/23. doi: 10.1371/journal.pone.0042902 PMID: 22912760

69. Ciannamea S, Kaufmann K, Frau M, Tonaco IA, Petersen K, Nielsen KK, et al. Protein interactions of MADS box transcription factors involved in flowering in Lolium perenne. J Exp Bot. 2006; 57(13):3419–31. Epub 2006/09/29. doi: 10.1093/jxb/erl144 PMID: 17005923

70. James P, Halladay J, Craig EA. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics. 1996; 144(4):1425–36. Epub 1996/12/01. PMID: 8978031

71. Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol. 2005; 139(1):5–17. doi: 10.1104/pp.105.063743 PMID: 16166256