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THE ROLE OF NUCLEAR FACTOR Y TRANSCRIPTION FACTORS IN PLANT DEVELOPMENT

A DISSERTATION APPROVED FOR THE
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This dissertation is dedicated to my wonderful family who supported me throughout the duration of this project, particularly my wife Emily and daughters Amy and Mia whom I couldn't have done this without, and in loving memory of my grandfather Frank Guerriero who always inspired me to be a better man.

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Abstract

Due to their sessile nature, plants must coordinate their growth and development based on the surrounding environmental conditions. This coordination is regulated by alterations in gene expression, leading to appropriate developmental outcomes. Transcription factors are instrumental in integrating environmental signals by modulating gene activity at the mRNA level. *NUCLEAR FACTOR Y* (NF-Y) is a hetrotrimeric transcription factor comprised of the subunits *NF-YA*, *NF-YB* and *NF-YC*. Although NF-Y are well studied in animal and fungal systems, little is know about their function in plants. Despite the paucity of genetically defined roles for NF-Y in plants, NF-Y has been implicated in a number of critical developmental processes.

Here I describe the overlapping and opposing functionality of multiple *NF-Y* genes in three developmental processes using reverse genetic approaches in *Arabidopsis thaliana*. In chapter two, candidate *NF-YC* genes regulating photoperiodic flowering were defined by assaying tissue specific expression patterns. Creating a triple mutant of *NF-YC3*, *NF-YC4* and *NF-YC9* revealed their overlapping functionality in stimulating flowering in response to inductive photoperiods. Further, NF-YC3, C4 and C9 were shown to physically interact with transcription factor CONSTANS, a well-known regulator of photoperiodic flowering. In addition, NF-Y are required for CONSTANS to activate its down stream target *FLOWERING LOCUS T*.

To determine *NF-YC* functions outside of flowering time, microarrays were performed on *NF-YC3*, *C4*, *C9* triple mutant plants. Analysis of the microarray data led to the hypothesis that *NF-Y* play a role in abscisic acid (ABA) signaling. Chapter three investigates this hypothesis. Phenotypic analysis of mutant combinations of *NF-YC3*, *C4* and *C9* demonstrate a clear role for these genes in determining germination timing in response to ABA. Unlike in flowering where *NF-YC3*, *C4* and *C9* have overlapping functionality, *NF-YC3* and *NF-YC9* have opposing role to *NF-YC4* in response to ABA. In addition, yeast two hybrid screens showed that NF-YC can interact with bZIP transcription factor involved in ABA responses.

Yeast two hybrid screens demonstrate that NF-Y not only interacts with CONSTANS but with many members of the CONSTANS-Like gene family. This led to the hypothesis that interactions between NF-Y and the CONSTANS-Like gene family are common, yet previous functional associations between these gene families were limited to the flowering time phenotype. One such example is my finding that CONSTANS-Like 3 physically interacts with NF-YC subunits. *CONSTANS-Like 3* has a demonstrated role in photomorphogenesis, suggesting that NF-YC proteins may also have a role in this process. In chapter four, *NF-Y* subunits are shown to also play a role in photomorphogenesis similar to that of *CONSTANS-Like 3*. These data imply that the NF-Y/CONSTANS-Like association extends beyond flowering and are likely to be widespread.

Through our understanding of NF-Y and their function we extend our knowledge of how plants adapt to their surroundings. Here NF-Y have been shown to play critical roles in three different developmental transitions: (1) seed dormancy to active growth, (2) from skotomorphogenic growth to photomorphogenic growth, (3) from vegetative to reproductive growth. Each of these transitions represents an important decision in the plant lifecycle, and their proper timing is critical for the ultimate success of the organism.

Chapter 1: Introduction

Portions of this Introduction Chapter, including the phylogenetic trees, are in press at The Plant Cell as part of a larger review article.

Petroni, K., Kumimoto, R.W., Gnesutta, N., Calvenzani, V., Fornari, N., Tonelli, C., Holt III, B.F., and Mantovani, R. (2012). The promiscuous life of plant NUCLEAR FACTOR Y (NF-Y) transcription factors. Plant Cell.

Introduction

The capacity of a plant to coordinate development with changing environmental conditions ensures the greatest chance for survival and reproductive success. Therefore, it is critical for plants to integrate these signals to induce physiological and developmental changes. To bring about these changes, careful regulation of gene activity is necessary. Transcription factors (TFs) are proteins that regulate gene activity at the mRNA level and consequently are required for proper growth and development at all stages of the plant lifecycle.

TFs exert their regulatory control by binding specific DNA sequences in the promoter regions of the genes to either activate or repress expression. Interestingly, plants have a greater complement of TF genes, as well as a greater diversity of TF families, in their genomes when compared to animals or fungi (Riechmann et al., 2000; Shiu et al., 2005). Due to their sessile nature, this led to the proposal that plants evolved additional TFs to cope with environmental conditions (Shiu et al., 2005). The expansion of the TF complement in plants is not strictly due to lineage specific transcription factor families – i.e., TFs that are only found in plants. In fact, the majority of TF families that are conserved between eukaryotic kingdoms are greatly expanded in plants (Shiu et al., 2005).

Nuclear Factor Y (NF-Y) TFs have been described in all sequenced eukaryotes to date. NF-Y TFs are composed of three proteins from unrelated families. The mature NF-Y transcription factor is comprised of one NF-YA, one NF-YB and one NF-YC subunit (Mantovani, 1999; Dolfini et al., 2012). NF-YB and NF-YC share sequence and structural similarity to histones H2A and H2B, respectively (Baxevanis et al., 1995). Like their histone relatives, NF-YB and NF-YC form a dimer with major contacts throughout the histone fold motifs (Romier et al., 2003). After initial formation in the cytoplasm the NF-YB/NF-YC dimer is imported to the nucleus where it associates with nuclear localized NF-YA (Frontini et al., 2004). Once in complex, NF-Y binds to the core *cis*-regulatory nucleotide sequence *CCAAT* where it can act as both a positive or negative regulator of transcription (Ceribelli et al., 2008; Donati et al., 2008).

NF-Ys were first characterized in animal systems where each is encoded at a single locus. NF-Y plays an indispensible role in animal systems, as mutations of the NF-YA subunit in both mouse and *Drosophila melanogaster* are lethal (Bhattacharya et al., 2003; Yoshioka et al., 2007). Transient inactivation of NF-Y in mouse systems led to defects in cell cycle control, possibly explaining the cause of lethality (Benatti et al., 2008). The fungi, *S. cerevisiae*, also contains single NF-Y fore each paralogous subunit, where they are annotated as HEME ACTIVATED PROTEINS (HAP2, HAP3 and HAP5) and regulate the yeasts' ability to grow on non-fermentable carbon sources (Pinkham et al., 1987; Hahn and Guarente, 1988; McNabb et al., 1995; McNabb et al., 1997). In addition to

the heterotrimeric complex seen in mammals, fungi have a fourth subunit called HAP4 that acts as a transcriptional activation domain when associated with the *CCAAT* binding HAP2/HAP3/HAP5 complex (Forsburg and Guarente, 1989). In plants and animals, there are no HAP4 paralogs and the transcriptional activation potential has been split between the NF-YA and NF-YC subunits (Li et al., 1992; Coustry et al., 1996; di Silvio et al., 1999).

In contrast to mammals and fungi, where typically a single gene encodes each NF-Y subunit, angiosperm genomes contain multiple genes for each subunit. For example, in Arabidopsis there are 10 *NF-YA*, 10 *NF-YB* and 10 *NF-YC* genes (Gusmaroli et al., 2001, 2002; Siefers et al., 2009; Laloum et al., 2012). Although each *NF-Y* family in Arabidopsis is relatively small, they can theoretically combine to form 1,000 unique TFs. Because of the expansion of these gene families in plants, functional over-lap between members has made genetic studies laborious. Consequently, no complete NF-Y complex has been genetically demonstrated for a single process. Although NF-Y studies are lagging in plants, NF-Ys have emerged as central players in a number of critical plant processes. Here the current state of research on NF-Y TFs in plants will be reviewed.

Classification and Expression of NF-Y subunits in Angiosperms

Systematic identification and annotation of *NF-Y* genes has been performed on the dicot *Arabidopsis thaliana* as well as the monocots *Oryza sativa* (Rice),

Triticum aestivum (wheat), and *Brachypodium distachyon (Gusmaroli et al., 2001, 2002; Stephenson et al., 2007; Thirumurugan et al., 2008; Siefers et al., 2009; Cao et al., 2011)*. In the examined plant species, *NF-YA*, *NF-YB* and *NF-YC* have gene complements ranging from 7 to 16 genes. Although each NF-Y family has been expanded in plants, the number of genes in any particular family varies between species. For example, Arabidopsis has 10 *NF-YC* genes while rice and Brachypodium have seven and nine, respectively.

The expansion of plant *NF-Y* gene families has led to the intimidating task of identifying biologically relevant NF-Y complexes. To start sorting through the many possible NF-Y complexes, several groups have characterized *NF-Y* mRNA expression patterns for diverse plant species. Organ specific expression patterns along with phylogenetic relationships have been established systematically by RT-PCR in *Arabidopsis thaliana*, *Oryza sativa*, *Brachypodium distachyon*, and *Triticum aestivum* (Gusmaroli et al., 2001, 2002; Stephenson et al., 2007; Thirumurugan et al., 2008; Cao et al., 2011b), although the *Triticum* phylogenetic classifications and database annotations still require further input to make these published analyses broadly useful. Based on current RT-PCR expression analyses, no strong patterns connecting NF-Y phylogenetics and expression emerge, but *NF-Y* expression can be broken down into two very general classes: genes that are broadly expressed (found in nearly all tissues tested) and genes with more restricted or organ specific expression patterns (Figures 1.1-1.3). To date, most NF-Y phenotypes

have been associated with the broadly expressed subunits - a notable exception being the narrowly expressed *LEAFY COTYLEDON* (*LEC*) class of *NF-YB*, where expression is restricted to the embryo (Figure 1.2).

In addition to RT-PCR data, tissue specific expression has also been examined for each of the *AtNF-Y* genes using promoter::glucuronidase (*pNF-Y::GUS*) reporter gene fusions (Siefers et al., 2009). These tissue specific expression patterns have already proven valuable for identifying NF-Y involved in photoperiod-dependent flowering (Kumimoto et al., 2010). Photoperiod dependent flowering (further discussed below) is regulated by interactions between NF-Y subunits and the floral promoting protein CONSTANS (CO). Because CO function in flowering time requires vascular expression in leaves (An et al., 2004), the *pNF-Y::GUS* reporter lines were instrumental for identifying three vascular expressed *AtNF-YC* necessary for CO-mediated control of photoperiod-dependent flowering .

Adding to surveys of organ and tissue specific expression patterns, several studies have systematically examined NF-Y responses to various environmental conditions and stresses (Stephenson et al., 2007, 2010, 2011; Hackenberg et al., 2012) and numerous studies have implicated NF-Y in regulating photosynthesis and drought responses (Kusnetsov et al., 1999; Miyoshi et al., 2003; Nelson et al., 2007; Li et al., 2008; Stephenson et al., 2010, 2011). Analysis of public microarray data also points to possible NF-Y

functions related to photosynthesis, flowering time, and drought. Analysis of cell specific transcriptome data from rice revealed the *CCAAT* -box element is associated with cell specific transcripts in both leaf (mesophyll, vein and primordia) and dehydration-response data sets (Jiao et al., 2009). Finally, up regulation of *Arabidopsis NF-Y* is correlated with leaf senescence (Breeze et al., 2011). Together these expression datasets, along with publically available microarrays, serve as a basic entrée point to *NF-Y* analyses and can be used to narrow down the number of candidates acting in a particular developmental stage or tissue type.

Functional Studies of NF-Y in plants

Gametophyte and Embryo Development

Like in animals, *NF-Ys* play a critical role in early development. Mutations of Arabidopsis *NF-YB9*, initially annotated *LEAFY COTYLEDON 1* (LEC1), lead to pleiotropic defects during embryo development including, reduced lipid body accumulation and reduced tolerance to desiccation which is necessary for proper seed dormancy. Arabidopsis *LEC1/NF-YB9* along with its close paralog *LEC-1-Like/NF-YB6* defined a novel, plant specific class of *NF-YB* required for proper embryo development (Kwong et al., 2003). The *LEC1* class of *NF-YB* have characteristic changes within the highly conserved Histone Fold Motif that have been demonstrated to be required for their function in embryogenesis (Kwong et al., 2003).

Orthologs of *Arabidopsis LEC1* and *LEC1-Like* have been identified in several plant species, including conifers, and the non-seed producing lycophytes (Xie et al., 2008). Functional studies of *LEC1-like NF-YB* genes in corn, rice, carrot, grapevine, and sunflower all support a conserved role in embryo development (Zhang et al., 2002; Fambrini et al., 2006; Yazawa and Kamada, 2007; Schellenbaum et al., 2008; Shen et al., 2010; Uddenberg et al., 2011). Because of the high degree of conservation of function and their ability to regulate seed oil content, *LEC1-like NF-YB* genes have been used to develop crops with enhanced seed oil content. Overexpression of *LEC1* orthologs in corn or canola can result in significant increases in seed oils, potentially adding value to these significant crops (Mu et al., 2008; Shen et al., 2010; Tan et al., 2011).

LEC1 and *LEC1-Like* can enter into complexes with Abscisic Acid Response Element (ABRE)-binding transcription factor bZIP67 to activate transcription of seed specific storage protein genes (Yamamoto et al., 2009a). This is an important result as it presents a direct link between *LEC1/NF-YB9* and abscisic acid (ABA) signalling - the primary phytohormone in establishing desiccation tolerance and dormancy in seeds. In addition, chromatin immunoprecipitation to chip experiments of LEC1/NF-YB9 showed an over-representation of the ABRE element in the promoters of LEC1 target genes (Junker et al., 2012). Adding validity to this study, the *CCAAT* element was also over-represented in the bound promoter elements.

NF-YA genes from Arabidopsis also have demonstrated roles in embryogenesis and fertilization. A large-scale screen to identify genes required for female gametophyte function identified a mutant of *nf-ya2* that shows unfertilized ovules while retaining normal pollen tube attraction to the egg cell (Pagnussat et al., 2005). Over-expression of *NF-YA1*, *NF-YA5*, *NF-YA6* and *NF-YA9* led to abnormal pollen, and arrest of embryo development at various stages. Adult plants recovered from these transgenic lines developed somatic embryos on vegetative tissues, similar to what is observed in *LEC1/NF-YB9* over-expression plants (Mu et al., 2012). Because loss of function mutants for *NF-YA1*, *A5*, *A6* and *A9* showed no measurable phenotypes in embryo development it remains to be seen if the over-expression phenotypes represent native function for these genes or neomorphic mutations. Disruption of an *NF-YA* gene from canola (*BnCBF-B*) using RNAi also led to reduced fertility of both the male and female gametophyte (Levesque-Lemay et al., 2003). *NF-Y* function in gametophyte function appears to be conserved in conifers where an *NF-YC* subunit from *Picea wilsonii* regulates the growth and guidance of the pollen tube (Yu et al., 2011).

Abiotic Stress and Nodulation

NF-Ys not only have connections to the stress related phytohormone ABA during embryo development, but also in the adult plant. In Arabidopsis *NF-YA5* is up regulated by ABA and osmotic stresses, and over-expression of *NF-YA5* confers drought tolerance (Li et al., 2008a). In addition, an *nf-ya5* loss of

function mutant is drought sensitive. *NF-YA5* and the entirety of the NF-YA family in plants are regulated post transcriptionally by the microRNA 169 (miR169) family (Jones-Rhoades and Bartel, 2004). Interestingly, while the miR169 family is conserved between monocots and dicots, their expression in response to stress seems to have diverged. In Arabidopsis miRNA169 is down regulated in response to drought (Li et al., 2008a). This is in contrast to rice where osmotic stress caused by salt resulted in miRNA169 up-regulation (Zhao et al., 2009). This observation is supported by two studies that systematically assayed *NF-Y* mRNA expression under drought conditions in wheat and Arabidopsis (Stephenson et al., 2007; Hackenberg et al., 2012). In the dicot Arabidopsis the majority of *NF-Y* genes were up regulated by drought stress. The opposite was true in wheat where *NF-Y* were down regulated by drought stress, indicating a possible functional divergence of *NF-Y* in response to osmotic stress between monocots and dicots.

In multiple plant species, over-expression of NF-YB proteins can also confer drought tolerance. Transgenic Arabidopsis plants over-expressing *NF-YB1* show enhanced survival in drought assays. In corn, the expression of the ortholog of *NF-YB1* (*ZmNF-YB2*) led to greater tolerance and importantly enhanced grain yield under field drought conditions (Nelson et al., 2007). Although studies of *NF-YA* subunits during stress implicate *NF-Y* as being part of the ABA mediated stress tolerance pathway (Li et al., 2008a), Affymetrix microarray analysis of Arabidopsis *NF-YB1* over-expressing plants did not show

similarity in gene expression to ABA treated plants. This led to the hypothesis that *NF-YB1* acts in a novel pathway to affect drought tolerance (Nelson et al., 2007).

Complexes containing both NF-Y and bZIP proteins mediate responses to Endoplasmic Reticulum (ER) Stress and the Unfolded Protein Response (UPR) (Liu and Howell, 2010). This functional relationship between *bZIPs* and *NF-Y* is conserved in plants and animal systems. In animal systems the bZIP protein ATF6 binds the ER Stress Responsive Elements (ERSE) in target promoter to activate transcription (Yamamoto et al., 2004). ATF6 can only bind to the ERSE when NF-Y is bound to a proximal *CCAAT* box. This is also true in Arabidopsis where NF-YA4, NF-YB3 and NF-YC2 are proposed to form a complex with bZIP28 to regulate ERSE promoter containing genes during ER stress (Liu and Howell, 2010). Although interactions between NF-YA4, NF-YB3, NF-YC2 and bZIP28 occur *in vitro*, genetic evidence supporting that particular NF-Y complex in ER stress responses is still lacking.

Arabidopsis *NF-YC2* is rapidly and strongly up regulated at the transcriptional level by a number of stresses (Hackenberg et al., 2012). Although transcriptional responses of *NF-YC2* implicate a role for stress responses, loss of function analysis of *nf-yc2* mutants showed no differences to control plants during photooxidative stress (Hackenberg et al., 2012). This suggests other closely related *NF-YC* may have over-lapping function in this process. The

construction of higher order mutants will be necessary to fully describe *NF-Y* function in stress responses.

Light Responses and Photosynthesis

NF-Ys have demonstrated roles in light and photosynthesis related gene expression in multiple plant species. An early study showed Arabidopsis *NF-YC2* could bind to the promoter region of a spinach photosynthesis related gene (Kusnetsov et al., 1999). In addition, mutation of the *CCAAT* box within the promoter abolished binding of that NF-Y in electrophoretic mobility shift assays (Kusnetsov et al., 1999). In Rice, simultaneous knockdowns of *OsNF-YB2*, *OsNF-YB3* and *OsNF-YB4* expression by RNAi led to plants with abnormal chloroplast development and reduced chlorophyll content. In addition the rice mutants had reduced expression of *LIGHT HARVESTING CHLOROPHYLL A/B BINDING PROTEIN* (Miyoshi et al., 2003). Arabidopsis mutants of *NF-YB9/LEC1* and *NF-YA5* showed reduced expression of *Lhcb* in response to low fluence rate blue light (Warpeha et al., 2007). While down regulation of *NF-Y* subunits in Arabidopsis and rice led to impaired expression of photosynthesis related genes, overexpression of *TaNF-YB3* in wheat led to increases in chlorophyll content (Stephenson et al., 2011). In addition several wheat *NF-YC* subunits are regulated transcriptionally by light. In particular *TaNF-YC11* is co-expressed with a number of critical photosynthesis genes (Stephenson et al., 2010).

Nodulation, Nitrogen and Root growth

In the model legume *Medicago truncatula, MtNF-YA1* is required for the proper formation of the nitrogen-fixing nodule (Combier et al., 2006). *MtNF-YA1* is highly expressed in the nodule and belongs to a group of highly related clade of *NF-YA* that appear to only be present in legume species (Combier et al., 2006; Laloum et al., 2012). Like the *NF-YA* involved in stress responses described above, *MtNF-YA1* is regulated post transcriptionally by members of the miRNA169 family. In addition *MtNF-YA1* activity is negatively regulated by a small peptide that is the product of an alternative splice of its own 5' leading sequence (Combier et al., 2006; Combier et al., 2008). Although all plant *NF-YA* have an at least one intron in the 5' leading sequence, it remains to be seen if this is a general form of regulation for *NF-YA*s (Laloum et al., 2012). In the bean *Phaseolus vulgaris* modulation of the expression of *PvNF-YC1* affects nodulation. In addition to affecting the nodulation process, *PvNF-YC1* appears to be involved in the selection of the infecting strain of Rhizobium (Zanetti et al., 2011). *NF-Y* are not only involved in nitrogen responses in legumes. In Arabidopsis, where nodulation does not occur, over-expression of miR169, leading to down regulation of *NF-YA2*, *NF-YA3*, *NF-YA5* and *NF-YA8*, altered nitrogen response phenotypes (Zhao et al., 2011). *NF-Ys* also control root growth in Arabidopsis. Overexpression of *AtNF-YB2* enhances primary root elongation due to faster cell division and/or elongation (Ballif et al., 2011). It is interesting to speculate that *NF-Ys* role in nitrogen usage and root growth in

Arabidopsis are some how related to their derived role in nodule formation in legumes.

Flowering Time

Multiple *NF-Y* genes have a genetically demonstrated role in the transition to flowering under inductive long day photoperiods in Arabidopsis. For both *NF-YB* and *NF-YC,* several genes with overlapping function in flowering have been discovered. Single mutants of both *nf-yb2* and *nf-yb3* show mild delays in flowering under inductive long day conditions (Cai et al., 2007; Kumimoto et al., 2008). When *nf-yb2* and *nfyb3* are combined, double mutants plants are significantly later flowering than either of the singles and essentially non-responsive to inductive light conditions (Kumimoto et al., 2008). In addition over-expressing either *NF-YB2* or *NF-YB3* activates precocious flowering. Similar results were demonstrated for three *NF-YC* subunits. *NF-YC3*, *NF-YC4* and *NF-YC9* have overlapping functionality in flowering and only the generation of a triple mutant yielded a strong late flowering phenotype (Kumimoto et al., 2010). Importantly interactions between the above NF-YB and NF-YC proteins have been demonstrated *in vivo*. Subsequent studies showed that overexpression of either *NF-YC1* or *NF-YC2* could also drive early flowering, although no loss of function phenotypes in flowering were described (Hackenberg et al., 2012).

An important discovery was the fact that NF-YB and NF-YC subunits interact with the well-studied master regulator of photoperiodic flowering CONSTANS (CO) (Ben-Naim et al., 2006; Wenkel et al., 2006). *CONSTANS* is a transcription factor that activates the florigen gene, *FLOWERING LOCUS T* (FT) (Suarez-Lopez et al., 2001; An et al., 2004; Corbesier et al., 2007). As would be predicted by its interaction with CO, NF-Y also regulates the expression of *FT* (Wenkel et al., 2006; Kumimoto et al., 2008; Kumimoto et al., 2010). Genetic studies have shown that *NF-Ys* are required for *CO* activity. *CO* is unable to drive flowering when over-expressed in either the *nf-yb2/b3* double or *nf-yc3/c4/c9* triple mutants (Kumimoto et al., 2010; Tiwari et al., 2010). The converse is also true, as over-expression of *NF-YB2* does not drive flowering in a *co* mutant background (Tiwari et al., 2012). Interestingly, over-expression of *NF-YB2* fused to a potent transcriptional activation domain can induce flowering in the absence of *CO* (Tiwari et al., 2012). One hypothesis is that NF-Y acts as a binding platform for CO which itself contains a potent transcriptional activation domain. This would be similar to how NF-Y function in yeast, where HAP2/HAP3/HAP5 bind DNA and HAP4 provides the transcriptional activation domain. However, CO was recently demonstrated to bind DNA in the *FT* promoter directly (Tiwari et al., 2010). A more appropriate model for this situation may be the relationship *NF-Y* has with *ATF6* during ER stress (see above). During ER stress NF-Y must be bound to the *CCAAT* -box before ATF6 can bind. The same may be true for NF-Y and CO, where NF-Y must bind DNA before CO is able to perform its function.

Examples of *NF-Y* in flowering extend beyond the Arabidopsis model. Several alleles of *OsNF-YB11*, a paralog of Arabidopsis *NF-YB2* and *NF-YB3* (figure2), have been identified as major quantitative trait loci for flowering in rice (Wei et al., 2010; Shibaya et al., 2011; Yan et al., 2011). Paralogs of *AtNF-YB2/3*, from *Brachypodium* (Cao et al., 2011) and barley (Liang et al., 2012), can drive early flowering when over-expressed in Arabidopsis. Further, *BdNF-YB6* overexpression could rescue late flowering Arabidopsis *nf-yb2 nf-yb3* mutants (Cao et al., 2011). In wheat, NF-Y and CO-LIKE (COL) genes interact to co-ordinately regulate flowering in response to vernalization and photoperiod (Distelfeld et al., 2009a; Li et al., 2011). Interestingly, the wheat COL protein VRN2 can compete with the NF-YA for interactions with the NF-YB/NF-YC dimer adding another layer of complexity to the NF-Y/COL complexes (see below). Even with possible mechanistic differences, these data suggest a strong conservation of the *NF-Y/CO* module affecting flowering time across the plant kingdom.

NF-Y protein interaction

To date, no complete *in-vivo* NF-Y complex in plants has been identified. Systematic studies of all possible NF-YB by NF-YC interactions show that these subunits interact promiscuously with essentially all combinations showing some level of interaction (Hackenberg et al., 2011; Calvenzani et al., 2012). More limited studies of the NF-Y trimer formation yielded similar results, with all

tested combinations interacting to varying extents (Calvenzani et al., 2012). This is not surprising as plant NF-Ys can also form functional DNA complexes with human and yeast subunits (Kumimoto et al., 2008; Calvenzani et al., 2012). Further study *in planta* will be necessary to validate functional NF-Y complexes.

Physical interactions between NF-Y and other TFs are emerging as important regulatory modules. Plant NF-Ys have been shown to interact with two different bZIPs (bZIP67 and bZIP28) in distinct pathways, with functions in gene regulation during embryo development and ER stress (Yamamoto et al., 2009a; Liu and Howell, 2010). As mentioned above, the association of bZIPs and NF-Ys appear across kingdoms, with examples in yeast and mammals (Yoshida et al., 2001; Yamamoto et al., 2004; Hortschansky et al., 2007). The bZIP family in Arabidopsis has approximately 70 members creating another possible layer of complex diversity for *NF-Y* related processes.

NF-Y also interacts with COL TFs that are in part defined by the presence by a conseverd 43-amino acid stretch named the *CO*, *COL*, and *TOC1* (CCT) domain. Wenkel *et. al*. made an important observation when they discovered the CCT domain showed high homology to the conserved DNA binding in the NF-YA subunit. This led to the hypothesis that CCT proteins, such as CO, could replace the NF-YA subunit in the NF-Y complex and bind DNA (Wenkel et al., 2006). At the time, the ability of CO to bind DNA was still in question. Although

it appears that CCT proteins can bind DNA independently of NF-Y, the ability for NF-Y and CCT proteins to compete for the NF-YB/NF-YC dimer has been confirmed using yeast 3 hybrid assays between the wheat NF-Y and CCT proteins (Li et al., 2011). Whether the trimeric complex containing NF-YB, NF-YC and a CCT forms *in vivo* or if it can bind DNA has yet to be determined.

One other study indicates that non-canonical NF-Y complexes using the NF-YB/YC dimer can be formed in plants. In rice a MADS-box TF, *OsMADS18*, can enter into a complex with *OsNF-YB1* (Masiero et al., 2002). *OsNF-YB1* has a naturally occurring amino acid substitution in the histone fold motif that precludes the NF-YA subunit from binding. This case supports the fact that NF-YB/YC dimer may be co-opted by other TFs, further expanding their already impressive complex diversity.

Although the NF-YB/YC/CCT trimeric complex has been demonstrated *in vitro,* recent genetic and biochemical evidence argues against this being the situation *in planta*. First the CCT domain protein CO was shown to bind DNA independently of NF-Y to a novel promoter element (Tiwari et al., 2010). Second, NF-YB subunits chimerically fused to a potent activation domain are able to drive flowering in the absence of *CONSTANS* (Tiwari et al., 2012). These two pieces of data suggest a co-operative model between NF-Y and CCT proteins similar to what is described with bZIP proteins (see above). To

sort out these possibilities, further studies of how these proteins enter into complexes and bind DNA are surely necessary.

Two additional, non-TF, NF-Y interacting proteins have been identified. Arabidopsis NF-YB9/LEC1 can physically interact with pirin1, a member of the iron containing cupin super family. This interaction is proposed to play a role in G-protein mediated cell signalling involved in ABA and germination responses (Warpeha et al., 2007). An NF-YC subunit from *Picea wilsonii* interacts with FKBP12 (Yu et al., 2011), a member of a large family, identified as targets of the immunosuppressive FK506 drug. These proteins have a multitude of cellular functions in both mammals and plants. How either of these proteins regulate NF-Y function is yet to be determined, although phenotypic analysis suggest they do function in the same pathways.

Conclusion and Perspective

Considering the amount of demonstrated functional overlap within the NF-Y families, remarkable progress been made in determining NF-Y function. Although the function of many NF-Y have been established, how NF-Y themselves are regulated remains to be determined. The primary outstanding issue going forward is that no complete heterotrimeric complex has been determined in plants. This limitation hinders the multitude of interesting questions regarding these TFs. One would assume that with ~1000 possible functional NF-Y combinations that each would have a different affinity for

promoter sequences. Partially because no NF-Y trimer has been described, very little is known about the promoter element NF-Y bind *in planta*. The first step in identifying targets for NF-Y was recently reported. Junker *et.al.* performed chromatin immunopreciptation (ChIP) to chip experiments with NF-YB9/LEC1. Several interesting targets were identified and validated through ChIP PCR experiments. It will be interesting to see this study extended by using other NF-YB proteins in the ChIP experiment and determining if the binding site preferences change.

The same argument of preference for DNA sequences could be made for transacting factors such as the bZIP and CCT proteins described above. A logical hypothesis would be that particular NF-Y would have a preference for specific interactions. This hypothesis already has support as Arabidopsis bZIP28 only interacts with NF-Y complexes containing NF-YB3 (Liu and Howell, 2010). A similar case is seen where bZIP67 only functionally interacts with NF-YB6 and NF-YB9 (Yamamoto et al., 2009a). This suggests that the bZIP interaction specificity is coordinated by the particular NF-YB subunit involved in forming the functional NF-Y trimer. To date, no such preferences have been shown for CCT proteins.

So far, very little is known about the regulation of each of the individual NF-Y subunits. Like the related core histones, NF-Y subunits are subject to multiple posttranslational modifications in animal systems. Both NF-YA and NF-YB can

be acetylated, in addition NF-YA can be phosphorylated and ubiquitinated (Currie, 1997; Li et al., 1998; Yun et al., 2003; Manni et al., 2008). While these modifications affect NF-Y activity in animal systems, no posttranslational modifications have been reported in plants. Further study using the Yeast-2-Hybrid screen could identify factors responsible for these modifications in plants. Because of their multimeric nature, NF-Y transcription factor have the ability to read out several environmental conditions simultaneously, thus coordinating the response at a single locus. A greater understanding of how NF-Y complexes are formed and regulated as well as their DNA binding affinities will lead to a greater understanding of how plants can cope with a vast number of environmental situations.

The work provided here has begun to address some of these outstanding questions revolving around plant *NF-Y*. One focus of this research was to genetically verify an *in planta* NF-Y complex. Through this work *NF-YC* subunits involved in flowering were genetically defined leaving only the NF-YA subunits outstanding (Chapter 2). In addition, phenotypes for *NF-YC* were extended to ABA germination opening a new avenue to assess NF-Y targets (Chapter 3).

In addition, NF-Ys requirement for *CONSTANS* in flowering function were established (Chapter 2). Functional interaction of NF-Y and COL genes, beyond flowering time, were demonstrated for light regulated growth (Chapter 4).

Further evidence linking NF-Y to bZIP proteins in ABA related processes are also presented (Chapter 3). These studies not only extend our knowledge of how NF-Y function throughout plant development but lays the groundwork for future experiments.

Figures and Tables

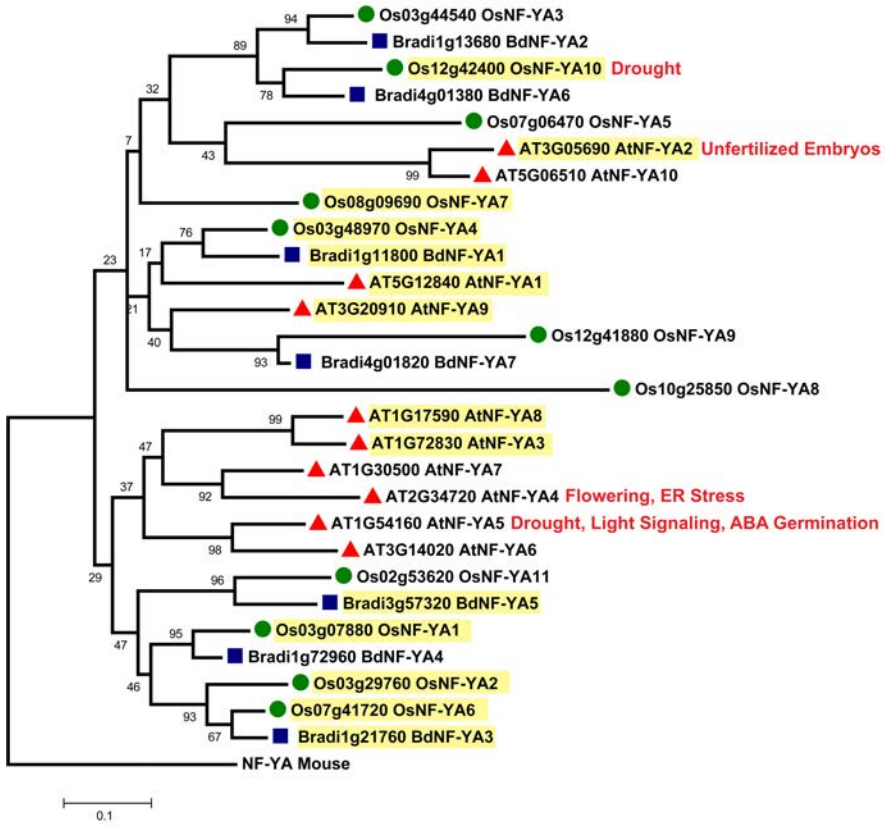

Figure 1.1. Phylogenetic tree of *Oryza sativa*, *Brachypodiun distachyon* and *Arabidopsis thaliana* NF-YA subunits genes. For full length NF-YA proteins, multiple sequence alignments were generated using ClustalW (Thompson et al., 2002) as implemented by Molecular Evolutionary Genetics Analysis (MEGA) software, version 5.0 (Tamura et al., 2011). Phylogenetic trees were constructed by neighbor-joining with complete deletions as implemented by MEGA. Reliability values at each branch represent bootstrap samples (5000 replicates). The mouse NF-YA was used to root the tree. Yellow highlighted genes are broadly expressed in all RT-PCR experiments. Documented phenotypes for a given gene are in red text.

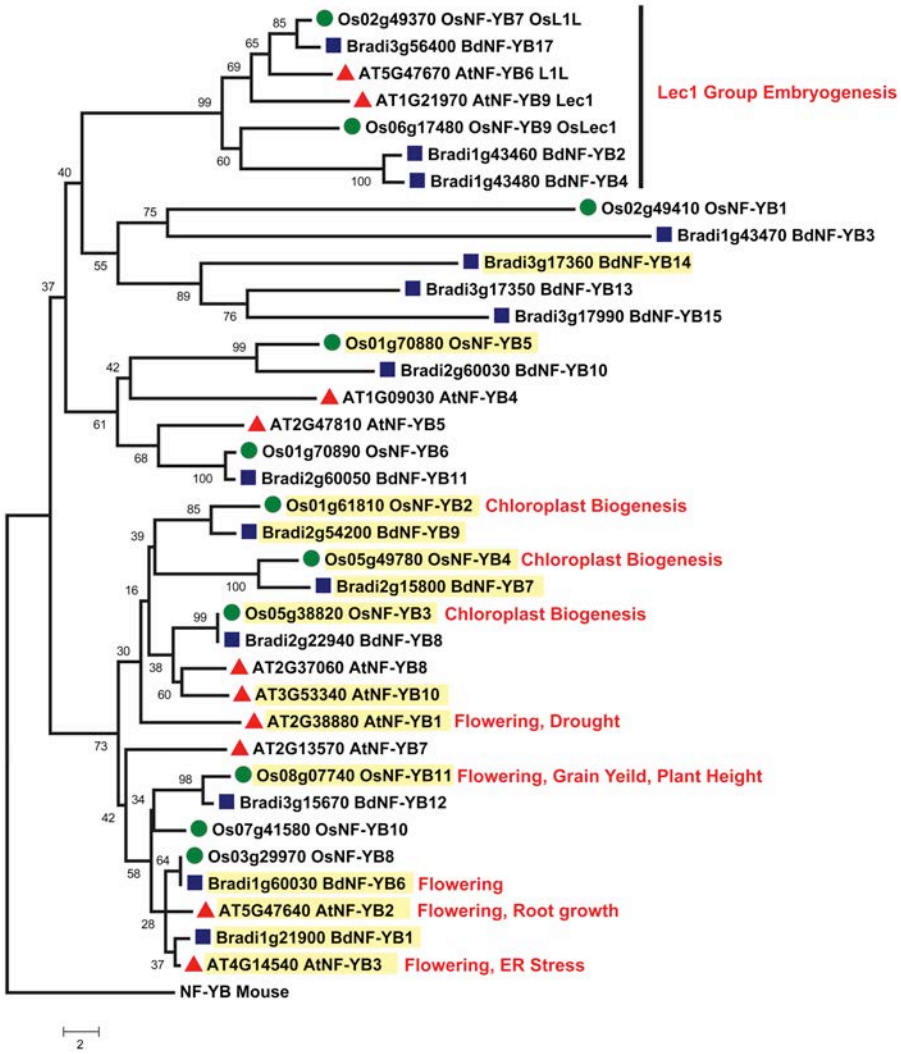

Figure 1.2. Phylogenetic tree of *Oryza sativa*, *Brachypodiun distachyon* and *Arabidopsis thaliana* NF-YB subunits genes. For full length NF-YA proteins, multiple sequence alignments were generated using ClustalW (Thompson et al., 2002) as implemented by Molecular Evolutionary Genetics Analysis (MEGA) software, version 5.0 (Tamura et al., 2011). Phylogenetic trees were constructed by neighbor-joining with complete deletions as implemented by MEGA. Reliability values at each branch represent bootstrap samples (5000 replicates). The mouse NF-YB was used to root the tree. Yellow highlighted genes are broadly expressed in all RT-PCR experiments. Documented phenotypes for a given gene are in red text.

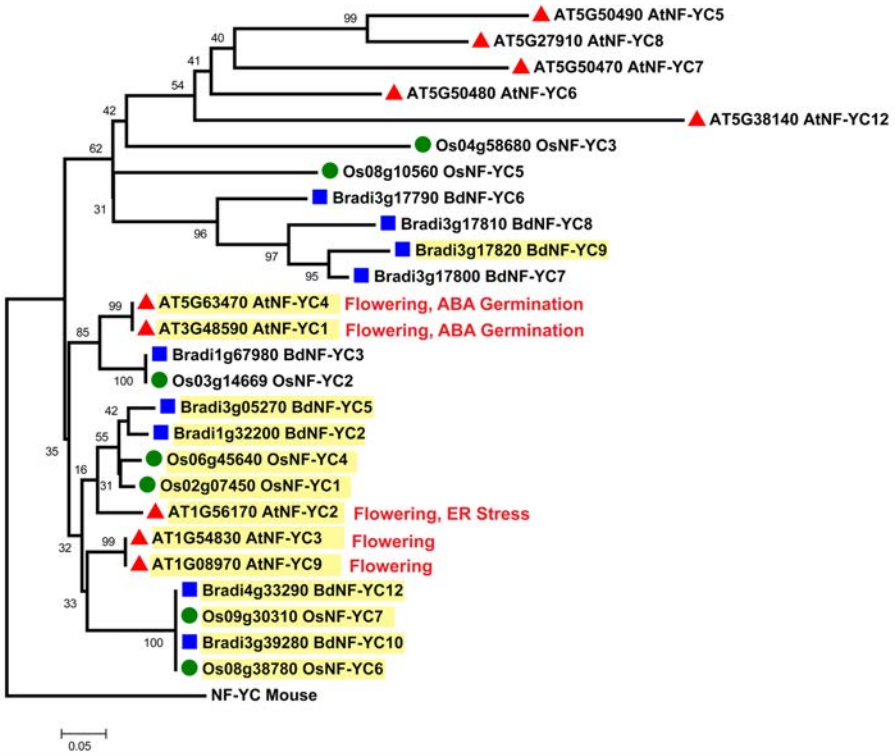

Figure 1.3. Phylogenetic tree of *Oryza sativa*, *Brachypodiun distachyon* and *Arabidopsis thaliana* NF-YC subunits genes. For full length NF-YA proteins, multiple sequence alignments were generated using ClustalW (Thompson et al., 2002) as implemented by Molecular Evolutionary Genetics Analysis (MEGA) software, version 5.0 (Tamura et al., 2011). Phylogenetic trees were constructed by neighbor-joining with complete deletions as implemented by MEGA. Reliability values at each branch represent bootstrap samples (5000 replicates). The mouse NF-YC was used to root the tree. Yellow highlighted genes are broadly expressed in all RT-PCR experiments. Documented phenotypes for a given gene are in red text.

Chapter 2: NF-YC3, NF-YC4, and NF-YC9 are required for CONSTANS-mediated, photoperiod-dependent flowering in *Arabidopsis thaliana*

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Summary

NF-Y transcription factors represent a complex of three proteins called NF-YA, NF-YB, and NF-YC. Each protein is highly conserved in eukaryotes and in the plant lineage has undergone numerous rounds of duplication. Individual NF-Y are emerging as important regulators of several essential plant processes, including embryogenesis, drought resistance, maintenance of meristems in nitrogen fixing nodules, and photoperiod-dependent flowering time. Building on the recent finding that NF-YB2 and NF-YB3 have overlapping functionality in Arabidopsis photoperiod-dependent flowering (Kumimoto et al., 2008), we have identified three NF-YC (NF-YC3, 4, and 9) that are also required for flowering and physically interact *in vivo* with both NF-YB2 and NF-YB3. Further, NF-YC3, 4, and 9 can physically interact with full length CONSTANS (CO) and are genetically required for CO-mediated floral promotion. Collectively, the present data greatly strengthens and extends the argument that CO utilizes NF-Y transcription factor complexes for activation of *FLOWERING LOCUS T* (*FT*) during photoperiod-dependent floral initiation.

Introduction

Many plant species have evolved to initiate flowering under specific photoperiods (Imaizumi and Kay, 2006; Kobayashi and Weigel, 2007). For example, flowering in the model plant *Arabidopsis thaliana* is rapidly induced in long photoperiods - i.e., *Arabidopsis* is a long day (LD) plant. Other photoperiod responsive species, such as the monocot rice (*Oryza sativa*), are induced to flower in short photoperiods (SD plants). In *Arabidopsis*, a key regulator of photoperiod-dependent flowering time is the zinc-finger type transcriptional activator *CONSTANS* (*CO,* (Redei, 1962; Koornneef et al., 1991; Putterill et al., 1995). *CO* mRNA levels are controlled by the circadian clock and oscillate on a daily basis – *CO* expression peaks during the day in LD conditions and during the night in SD conditions (Suarez-Lopez et al., 2001). Peak *CO* expression during the day is essential for CO activity because the protein is rapidly degraded in the dark (Valverde et al., 2004; Jang et al., 2008; Liu et al., 2008). Under LD conditions, CO protein accumulates and rapidly induces expression of *FLOWERING LOCUS T* (*FT*) (Samach et al., 2000). FT protein subsequently translocates to the meristem and triggers its conversion to a floral meristem (Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Tamaki et al., 2007).

Although CO is thought to directly control *FT* expression (Samach et al., 2000), precisely how CO might integrate with the *FT* promoter remains unclear. Potentially addressing this question, several recent publications demonstrated

that NF-Y transcription factors are intimately involved in photoperiod-dependent flowering. Research groups studying flowering time in both tomato (*Solanum lycopersicum*) and *Arabidopsis* identified NF-Y subunits as CO-interacting proteins (Ben-Naim et al., 2006; Wenkel et al., 2006). Following these initial descriptions of NF-Y/CO interactions, two independent research groups described the same *nf-yb2* loss-of-function allele as causing delays in flowering time (Cai et al., 2007; Chen et al., 2007). Conversely, overexpression of *NF-YB2* resulted in the opposite phenotype - significantly more rapid flowering. Finally, *nf-yb2 nf-yb3* double mutants phenocopied *co* mutants - i.e., late flowering and strong downregulation of *FT* expression (Kumimoto et al., 2008). Because mammalian and yeast NF-Ys are well-characterized as transcriptional-activating, DNA-binding complexes, these results strongly suggested that CO interacts with DNA via an NF-Y platform.

Genes encoding NF-Y transcription factors are found in all eukaryotes (Edwards et al., 1998; Maity and de Crombrugghe, 1998; Mantovani, 1999; Matuoka and Chen, 2002; Siefers et al., 2009). In mammals, NF-Y functions as a heterotrimer consisting of the single gene encoded subunits NF-YA, NF-YB, and NF-YC - individual NF-Y subunits do not appear to have DNA binding or transcriptional activation properties (Maity and de Crombrugghe, 1998). Mammalian NF-Y subunits assemble in a strict, stepwise fashion to form the mature, DNA-binding transcription factor (Maity et al., 1992; Sinha et al., 1996). Initially, the two histone-fold containing proteins, NF-YB and NF-YC, form a

dimer that translocates to the nucleus (Frontini et al., 2004; Steidl et al., 2004; Goda et al., 2005; Tuncher et al., 2005). Once in the nucleus, NF-YA is recruited and the resulting mature NF-Y transcription factor is competent to bind promoters at CCAAT nucleotide sequences. Recent bioinformatic analyses suggest that ~7.6% of all human promoters have functional CCAAT binding sites (FitzGerald et al., 2004).

In the plant lineage, the discovery of a complete NF-YA/B/C complex controlling the expression of a particular gene or process has never been described. Single plant *NF-YA* and *NF-YB* genes are known to have functions in embryogenesis, drought resistance, ABA signaling, nitrogen fixing nodule development, and flowering time (Lotan et al., 1998; Kwong et al., 2003; Combier et al., 2006; Nelson et al., 2007; Suzuki et al., 2007; Warpeha et al., 2007; Li et al., 2008b). To our knowledge, only one *NF-YC* loss of function phenotype has been reported in Arabidopsis - *nf-yc4* mutants have increased sensitivity to ABA in plate germination assays (Warpeha et al., 2007). The current lack of knowledge regarding complete NF-Y complexes in the plant lineage is most likely due to overlapping functionality - in Arabidopsis there are 10 NF-YA, 10 NF-YB, and 10 NF-YC (Gusmaroli et al., 2001, 2002; Siefers et al., 2009) and this expansion appears to be consistent in all sequenced monocots and dicots (Stephenson et al., 2007; Thirumurugan et al., 2008; Siefers et al., 2009).

In this paper we identify and describe three Arabidopsis *NF-YC* genes with overlapping functionality in flowering time. Further, we demonstrate that *CO* function in floral promotion is, at least, partially dependent on these genes. We present yeast two hybrid (Y2H) and *in vivo* interaction data demonstrating that these three NF-YC proteins physically interact with the known floral promoting NF-YB2 and NF-YB3 proteins. Further, as previously demonstrated for tomato COL1 (TCOL1, (Ben-Naim et al., 2006)), our data supports the general concept that CO and CO-LIKE (COL) proteins are recruited to NF-Y complexes via the NF-YC subunit.

Results

Identification of *NF-YC* candidate genes in flowering time regulation

In Arabidopsis, *NF-YC* represents a multi-gene family containing thirteen members (Figure 1A) (Siefers et al., 2009). Using yeast two-hybrid (Y2H) analyses, it was previously reported that eight different NF-YC (NF-YC1 through 7 and NF-YC9) can physically interact with the CCT (CO, CO-LIKE, and TIMING OF CAB1) domains of CONSTANS (CO) and/or CO-LIKE 15 (COL15, (Wenkel et al., 2006). Although this data clearly indicated the potential for NF-YC/CCT-domain interactions, we expected a more limited set of interactions *in vivo* - an expectation supported by the highly variable, tissue-specific expression patterns of *NF-YC* genes (Siefers et al., 2009). To identify likely candidates for involvement in the CO dependent induction of flowering, we performed tissue specific gene expression analyses on all 13 members of the

NF-YC family. Because CO induces *FT* expression in the phloem tissue of young, LD-grown leaves (An et al., 2004), we inferred that candidate *NF-YC* genes would also be expressed in the leaf vasculature at this developmental stage.

The promoter region (1,000 bp upstream of the ATG) for each *NF-YC* was used to drive *in planta* expression of a ß-glucuoronidase (GUS) reporter gene (Siefers et al., 2009). Stable transgenic lines were grown for 10 days under standard LD conditions (16hr light/8hr dark, 22C) and microscopically assayed for GUS expression in the vascular tissue. *NF-YC3*, *NF-YC4*, and *NF-YC9* were consistently and strongly expressed in the leaf vasculature (Figure 2.1B). These three *NF-YC* are also expressed in the leaf mesophyll and other tissues, where they likely participate in the transcriptional control of genes unrelated to flowering time regulation (Siefers et al., 2009). Phylogenetic analysis revealed that NF-YC3, NF-YC4, and NF-YC9 are very closely related (Figure 2.1A); in fact, NF-YC3 and NF-YC9 are 100% identical throughout their conserved histone fold motifs. Therefore, we considered *NF-YC3*, *4*, and *9* our best candidates for further investigations.

Arabidopsis requires *NF-YC3*, *NF-YC4*, and *NF-YC9* for proper timing of photoperiod induced flowering

We isolated stable, homozygous T-DNA insertion lines for *NF-YC3* (SALK_034838, *nf-yc3-1*), *NF-YC4* (SALK_032163, *nf-yc4-1*) and *NF-YC9*

(SALK_058903, *nf-yc9-1*). The T-DNA insertions in *nf-yc3-1*, *nf-yc4-1*, and *nf-yc9-1* are located ~299 base pairs (bp) upstream of the translational start, ~512 bp after the translational start in an annotated exon, and ~102 bp upstream of the translational start, respectively. To examine the effects of these T-DNA insertions, we developed native antibodies for NF-YC3 and NF-YC4 (Figure 1C, we do not have an antibody for NF-YC9). Based on protein blot analyses, *nf-yc3-1* and *nf-yc4-1* represent very strong knockdown alleles (we did occasionally detect NF-YC3 protein, but it never exceeded ~5% normal accumulation). For the T-DNA insertion in *nf-yc9-1*, we examined mRNA accumulation by quantitative, real time (qRT)-PCR and microarray analysis (see Figure 2.4). We estimate that *nf-yc9-1* retains ~30% normal *NF-YC9* expression. These are currently the strongest mutant alleles we have obtained for each gene and the only alleles discussed in this paper (hereafter discussed without allele designation).

Under standard LD conditions, none of the single mutants showed significant changes in flowering time compared to the parental ecotype control (Columbia (Col-0), Fig. 2.2B). Because NF-YC3, NF-YC4, and NF-YC9 have high amino acid identity, we hypothesized that functional overlap could be masking roles in flowering time regulation. To test this hypothesis, we created all three possible double mutant combinations. Under LD conditions, all three double mutants showed a mild delay in flowering. The double mutant for the closely related paralogs *NF-YC3* and *NF-YC9* was consistently later flowering than the other

combinations (Figure 2.2A-B). This apparent functional overlap was fully confirmed by the creation of *nf-yc3 nf-yc4 nf-yc9* triple mutants (hereafter "*nf-yc triple*"). By comparison to parental Col-0 (mean = 15.6 leaves at bolting), the *nf-yc triple* produced almost twice as many total leaves before flowering (mean = 28.6).

Although *nf-yc triple* mutants had significantly delayed flowering, they did not flower as late as either *co-sail* (Col-0 mutant allele of *CO*, mean = 51 total leaves in our LD conditions, data not shown) or *nf-yb2 nf-yb3* mutants (mean = 48.3 from (Kumimoto et al., 2008). The simplest explanation is that the *nf-yc triple* still had residual expression of *NF-YC9*, generating enough functional NF-YC9 to prevent stronger delays. Nevertheless, we cannot exclude the possibility that other *NF-YC* genes are involved in flowering time regulation.

To confirm that the observed flowering time phenotypes were due to the respective *NF-YC* T-DNA insertions, we performed complementation experiments. Constructs that expressed *NF-YC3*, *4* and *9* from their native promoters were intergrated into the *nf-yc triple* by Agrobacterium mediated transformation (Bechtold et al., 1993). Transformants were selected by resistance to glufosinate ammonium (BASTA). Each construct was able to rescue the *nf-yc triple* late flowering phenotype (Figure 2.2C, T2 generation plants shown). For all three genes, >50% of the original BASTA resistant T1 lines rescued the late flowering phenotype.

***NF-YC3*, *4*, and *9* are primarily involved in photoperiod dependent flowering**

Previous research demonstrated that the roles for *NF-YB2* and *NF-YB3* in flowering time appear to be primarily confined to the photoperiod dependent pathway (Kumimoto et al., 2008). To determine if *NF-YC3*, *4*, and *9* are required in the autonomous and vernalization pathways, flowering time for *nf-yc* mutants was quantified under SD conditions (8 hrs light/16 hrs dark) and after extended cold treatments (4C for 10 weeks). Under SD conditions, *nf-yc triple* mutants sometimes flowered a few leaves earlier than parental Col-0 (Figure 2.3A), but these differences were not consistently reproducible (e.g., see mock treated Col-0 versus *nf-yc triple* comparison in Figure 2.3C). In the vernalization experiments, *nf-yc* mutants flower later than Col-0, but are similarly responsive to the vernalization treatments - e.g., Col-0 flowered 21% earlier after vernalization compared to 29% earlier for the *nf-yc triple* mutant (Figure 2.3B).

We additionally determined if *NF-YC3*, *4*, and *9* were involved in the gibberellic acid (GA) flowering pathway. Col-0 and *nf-yc triples* were either mock or GA_3 treated in both SD and LD photoperiods. For SD experiments, plants were sprayed weekly with 100μM GA_3 until bolting (Wilson et al., 1992). For LD experiments, plants were germinated and grown on 10μM GA_3 plates for six days, transferred to soil, and sprayed one additional time with 100μM GA_3 at day 8. In both SD and LD conditions (Figure 2.3C), *nf-yc triple* mutants were

highly responsive to GA treatment (similar to Col-0). In SD conditions, *nf-yc triple* mutants flowered earlier than Col-0 after GA spray - this difference was statistically significant in some experiments (shown), but only represented a trend towards early flowering in other experiments. This suggests that *nf-yc triple* mutants may have slightly enhanced sensitivity to externally applied GA. Overall, the flowering time experiments are consistent with a predominant *NF-Y* role in the photoperiod dependant flowering pathway.

NF-YC regulate *FT* transcript expression

Previous research demonstrated that *NF-YB2 and NF-YB3* have additive roles in the positive regulation of *FT* expression (Kumimoto et al., 2008). To confirm a similar role for *NF-YC3*, *4*, and *9*, we examined the comparative expression of *FT* and *CO* in Col-0, *nf-yc triple*, and *co-sail* genetic backgrounds over a 24 hour time course. *CO* expression was similar between Col-0 and *nf-yc triple*, although we note that slightly higher *CO* levels in late day time points for *nf-yc triple* mutants were a consistent trend (Figure 2.4A). This data demonstrates that reductions in *CO* were not responsible for the measured delays in *nf-yc triple* flowering time. As consistently reported for LD-grown Arabidopsis (Imaizumi and Kay, 2006), *FT* expression was maximal approximately 16 hours after lights on (Figure 2.4B). *FT* expression was significantly repressed in both the *nf-yc triple* (~2 fold) and *co-sail* (essentially “off”) mutants. Intermediate levels of *FT* expression (between wild type and *co-sail*) in the *nf-yc triple*

mutants were consistent with both residual *NF-YC9* expression (Figure 2.4E) and the intermediate flowering delays.

To test the possibility that *NF-YC* genes are regulated by CO, we also measured transcript levels of *NF-YC3*, *4* and *9* in Col-0 compared to *nf-yc triple* and *co-sail* mutants over the time course. By comparison to parental Col-0, none of the *NF-YC* were significantly misregulated in *co-sail* and all three *NF-YC* were strongly downregulated in *nf-yc triple* plants (Figure 2.4C-E). All three *NF-YC* were most highly expressed in late day to night time points, with sharp increases beginning between 12-16 hours after lights on (*NF-YC4* had an additional peak at 4 hrs). It is important to note that these *NF-YC* are expressed both in and outside of the vascular tissue; therefore, this data represents entire above ground plants and may or may not accurately represent what happens in the vascular tissue. Collectively, the qRT-PCR data supports the hypothesis that *NF-YC3*, *4*, and *9* act as upstream regulators of *FT* expression. This data supports the hypothesis that NF-YC3, 4, and 9 act in a complex with NF-YB2, 3, and CO to positively regulate *FT* expression.

CO requires *NF-YC3*, *4*, and *9* to drive early flowering

All available evidence indicates that specific *NF-Y* are required for normal *FT* expression and photoperiod dependent flowering. The molecular and developmental phenotypes of various *nf-y* mutants appear to be virtually identical to those previously published for various *co* alleles (Robson et al.,

2001). Nevertheless, there is no available genetic evidence that *CO* function in floral promotion requires *NF-Y*. To provide this genetic evidence, we transformed Col-0 and *nf-yc triple* with a *CO* overexpression construct (*35S:CO-YFP/HA)* and measured total leaves at flowering. We hypothesized that constitutively overexpressed *CO* would drive early flowering when transformed into Col-0, but not *nf-yc triple*.

To establish an unbiased view of the range of possible phenotypes, we initially examined phenotypes of first generation (T1) transformants. Col-0 and *nf-yc triple* mutant control plants flowered at 13.4 (± 0.79) and 23.2 (± 2.27) total leaves, respectively (Figure 2.5A-B). When *35S:CO-YFP/HA* was introduced into Col-0, we observed a bimodal distribution of flowering times. As expected, the majority of lines (2/3) flowered significantly earlier than wild-type controls, while a smaller subset flowered later than Col-0 (Figure 2.5C). After repeating this analysis several times, we consistently measured this bimodal distribution (we suspect that late flowering lines are due to gene silencing, but this has not been confirmed in T1 plants. However, we have confirmed high rates for *35S:CO* silencing in subsequent generations). Significantly, the same *35S:CO-YFP/HA* construct was unable to drive early flowering phenotypes in the *nf-yc triple* mutant (Figure 2.5D, early flowering classes are completely missing from the frequency distribution). On average, *35S:CO-YFP/HA nf-yc triple* mutants did flower slightly earlier than *nf-yc triple* mutants (mean = 21.2 ± 5.4), but this difference was not statistically significant ($p>0.05$, ANOVA). This non-

significant, but likely biologically relevant decrease in total leaf number is consistent with residual *NF-YC9* accumulation in the *nf-yc triple*.

To extend the T1 results, we generated two independent, homozygous (T3), single insertion *35S:CO-YFP/HA nf-yc triple* lines (*35S:CO-YFP/HA nf-yc triple-16* and *-100*). We compared these two lines to stably overexpressed *CO* in the parental background (*35S:CO-YFP/HA-1* and *-202*) for three variables - *CO* mRNA expression, *FT* mRNA expression, and flowering time. Regardless of genetic background, all chosen *35S:CO* lines had elevated *CO* expression (Figure 2.6A). For *35S:CO* in the wild type background, elevated *CO* levels translated to the expected elevated *FT* levels (Figure 2.6B). For both *35S:CO nf-yc triple* lines, *FT* levels did not significantly change relative to the depressed levels in the *nf-yc triple* parental line - in fact, expression of *FT* remained below the levels of non-transgenic, parental Col-0 plants. Predictably, these *35S:CO nf-yc triple* mutants also maintained the late flowering phenotype of the parental *nf-yc triple* (Figure 2.6C-D).

Because *35S:CO-YFP/HA nf-yc triple-16* and *-100* had lower *CO* overexpression than *35S:CO-YFP/HA-1* and *202,* we additionally performed test crosses to parental Col-0 to demonstrate that these transgene insertions could drive early flowering in the context of functional NF-YC3, 4, and 9. Early flowering was readily measured in the resulting *35S:CO-YFP/HA/- NF-YC3/nf-yc3-1 NF-YC4/nf-yc4-1 NF-YC9/nf-yc9-1* genotype plants (Supplemental Figure

2.1). Thus, *35S:CO* overexpression could not rescue either low *FT* expression or the late flowering phenotypes of *nf-yc triple* mutants. This data strongly supports the conclusion that *NF-YC3*, *4*, and *9* are necessary for *CO* function.

NF-YC3, NF-YC4, and NF-YC9 physically interact with NF-YB2, NF-YB3, and CO

Yeast two hybrid studies previously revealed interactions between the CCT domain of CO and various NF-YB and NF-YC subunits. Relative to NF-Y with genetically demonstrated roles in flowering time, Wenkel et al. (2006) reported that NF-YB2, but not NF-YB3, could interact with the CCT domain of CO. Additionally, the CCT domain of CO interacted with NF-YC3 and 9, but not NF-YC4. Ben-Naim et al. (2006) also found that THAP5c (tomato homolog of NF-YC9) interacted with full length tomato COL1 and Arabidopsis CO. We wished to confirm and extend these findings to full length Arabidopsis CO for the entire suite of NF-Y with genetically demonstrated roles in photoperiod dependent flowering - NF-YB2 and 3 and NF-YC3,4, and 9. We also tested *in vivo* interactions between the NF-YB and NF-YC proteins.

We initially examined Y2H interactions between NF-YB2/3 and NF-YC3/4/9. Both NF-YB2 and 3 interacted strongly with NF-YC3, 4, and 9 (Figure 2.7A). Y2H interactions worked equally well regardless of which subunit was fused to the Gal4 DNA-binding (DBD) or activation domain (AD), although DBD:NF-YC3 fusions had low to moderate autoactivation (data not shown). To test whether

these interactions occur *in vivo*, we performed co-immunoprecipitation experiments. Initially, we transformed Col-0 with *35S:NF-YB2-* or *35SNF-YB3-YFP/HA* constructs. In agreement with previous studies, both constructs were able to drive early flowering under LD conditions (data not shown, (Cai et al., 2007; Kumimoto et al., 2008)). Total protein extracts were then collected from 10 day old seedlings and NF-YB2 or NF-YB3 were isolated by immunoprecipitation with HA antibodies. Co-immunoprecipitation of NF-YC3 and 4 was assayed by western blot analysis using native antibodies. NF-YB2 and 3 were readily able to co-immunoprecipitate NF-YC3 and 4 from total plant protein extracts (Figure 2.7B). Although we do not currently have an antibody for NF-YC9, all available genetic and Y2H data strongly suggests it should also be a component of these floral promoting, *in vivo* complexes.

To examine NF-Y by CO interactions, we created both full length DBD:CO and AD:CO constructs. Because DBD:CO constructs strongly activated all reporter genes in our Y2H system (ProQuest, Invitrogen), we examined DBD:NF-Y by AD:CO interactions (Figure 2.7C-D). In the context of a URA3 reporter gene, the previously described, moderate NF-YC3 autoactivation was not a problem for this analysis (Figure 2.7C). Using full length CO, our results differed from Wenkel et al. (2006) in that we consistently detected no interaction with either NF-YB2 or 3 (Figure 2.7D). This was true for three different reporter genes (Supplemental Figure 2.2). Additionally, we found that full length CO interacted strongly and consistently with all three NF-YC proteins (Figure 2.7D).

Because we could readily detect NF-YB2/3 by NF-YC3/4/9 interactions (Figure 2.7A), our DBD:NF-YB constructs appeared to be functioning properly. To further examine possible NF-YB and NF-YC interactions with CO/COL proteins, we performed fairly extensive library screening with DBD:NF-YB2, 3 and DBD:NF-YC9. For NF-YC9 we sequenced 167 putative interacting clones and found that 135 (81%) represented various NF-YB expressing clones. Of the remaining 32 interactors, 22 (13%) were COL family members. Identical, albeit less extensive, library screening with NF-YB2 and 3 did not identify any COL family members, although 24/51 (47%) putative interacting proteins represented various NF-YC. Therefore, from the pool of NF-YC interacting COL proteins, we chose three (COL3, 5, and 13) to directly test for Y2H interactions with NF-YB2 and 3 (Supplemental Figure 2.2). Once again, we did not detect any NF-YB2 or 3 interaction with CO or these related family members, although we readily detected interactions with appropriate controls. Collectively, our data suggest that all of the floral promoting NF-YB and NF-YC subunits can form complexes *in vivo* and that CO is most likely recruited into these complexes by direct interaction with the NF-YC components. Nevertheless, we cannot rule out the possibility that, in the context of a plant cell, NF-YB2 and 3 might interact with COL family members.

NF-YC3/4/9 interaction with CO primarily involves *FT* regulation

NF-Y and CO physically interact and have a clear genetic role in *FT* transcriptional control. To identify other possible targets of the NF-YC3/4/9-CO transcriptional complex, we conducted Affymetrix microarray experiments and compared the expression profiles of 10-day-old, whole, aboveground, LD-grown seedlings for Col-0, *nf-yc triple*, and *co-sail* genetic backgrounds (triplicate biological replicates for each background).

Compared to parental controls (Col-0), the *nf-yc triple* mutant had 5 upregulated and 6 downregulated genes (misregulation defined as expression difference >1.5 fold, Benjamini-Hochberg false discovery rate corrected ANOVA, $p \leq 0.05$). Using Benjamini-Hochberg corrected P value calculations, only one gene was simultaneously downregulated in *nf-yc triple* and *co-sail* mutants - *FT*. No genes were found to overlap in the *nf-yc triple* and *co-sail* upregulated sets. We note that the Benjamini-Hochberg correction is fairly conservative, potentially resulting in false negatives.

Discussion

We have provided evidence that *NF-YC3*, *NF-YC4*, and *NF-YC9* are additively necessary for proper photoperiod-dependant induction of flowering in *Arabidopsis thaliana*. Further, *CO* function in the transcriptional activation of *FT* requires these *NF-YC*. It was previously shown that *NF-YB2* and *NF-YB3* additively regulate *FT* expression and are required for photoperiod-dependent

initiation of flowering (Cai et al., 2007; Kumimoto et al., 2008). All three NF-YC proteins described here can physically interact with both floral promoting NF-YB proteins, establishing *at least* 6 possible complexes for CO-mediated activation of *FT* expression.

Perhaps as a response to various environmental conditions, such as water and nutrient availability, NF-Y combinatorial diversity could provide unique platforms for the fine-tuning of flowering time and other processes. The fact that NF-Y complexes are also involved in drought resistance (Nelson et al., 2007; Li et al., 2008b) further hints at a potential interaction between various environmental conditions and developmental outcomes. In addition to subtle changes in the positive activation of flowering time, we propose that NF-Y subunit heterogeneity at a given promoter might also provide antagonistic gene regulation - i.e., there may be both positive and negative NF-Y complexes competing for regulation of the same promoter.

In the plant lineage, there is extensive NF-Y heterogeneity and, by extension, many possibilities for differential regulation at a single DNA binding location. For example, while reported loss of function phenotypes suggest that NF-Y are positive regulators of *FT* expression (here and (Kumimoto et al., 2008)), it was also previously demonstrated that overexpression of NF-YA1, NF-YA4, and NF-YB1 reduced *FT* expression and delayed flowering (Wenkel et al., 2006; Nelson et al., 2007). Therefore, a subset of NF-Y are predicted to positively regulate *FT*

expression, while another subset can act as negative regulators. One trivial explanation is that overexpression of a subset of *NF-Y* leads to dominant negative phenotypes - i.e., these particular NF-Y family members are not normally involved in flowering and, when ectopically expressed, can interfere with floral promoting complexes. Whether or not these *NF-Y* overexpression phenotpes are biologically relevant, it is clear that *NF-Y* have the ability to both positively and negatively regulate *FT*. What role CO plays in these complexes remains unclear.

CO belongs to a plant specific, approximately 35 member family characterized by the CCT (CO, CO-LIKE, and TIMING OF CAB1) domain. Recent studies suggested that CO can physically interact with both NF-YB and NF-YC proteins via the CCT domain (Ben-Naim et al., 2006; Wenkel et al., 2006). In the present experiments, we were unable to replicate the CO/NF-YB2 interaction (Wenkel et al., 2006), but we were also examining full length proteins (as opposed to the CCT domain of CO). Thus, it remains possible that we do not detect interactions between NF-YB2 and full length CO in our Y2H system because appropriate conformational changes in CO only take place in the plant cell. Regardless of this possibility, we clearly and consistently measured interactions between full length CO and NF-YC3, NF-YC4, and NF-YC9. In yeast chromatin immunoprecipitation experiments, Ben-Naim et al. (2006) demonstrate that HAP5 (from tomato or yeast) is required to recruit TCOL1 to canonical yeast

CCAAT-containing promoters. The simplest, although not only, explanation for these data is that CO is recruited to NF-Y trimers via the NF-YC subunit.

Interestingly, CCT domains show amino acid sequence similarity to the DNA binding domain of NF-YA proteins (Wenkel et al., 2006; Distelfeld et al., 2009a; Distelfeld et al., 2009b; Siefers et al., 2009). This sequence similarity, coupled with the finding that NF-YA overexpression can cause late flowering, led Wenkel et. al. (2006) to speculate that CO and NF-YA compete for the occupancy of NF-YB/C dimers. In theory, this would suggest floral inhibiting NF-YA/B/C complexes as antagonists of floral promoting CO/NF-YB/C complexes.

Co-opting NF-YB/C dimers for novel functions might be a common outcome in the plant lineage. For example, the rice (*Oryza sativa*) MADS box domain protein OsMADS18 interacts with OsNF-YB1 and OsNF-YB1/NF-YC dimers (Masiero et al., 2002). Due to a naturally occurring point mutation, OsNF-YB1 containing dimers cannot interact with NF-YA proteins. Thus, OsMADS18/OsNF-YB1/NF-YC trimeric complexes also cannot bind CCAAT box sequences (Masiero et al., 2002). Likewise, CO/NF-YB/C trimeric complexes would not be expected to bind CCAAT sequences in the *FT* promoter. This is because essential histidine residues necessary for NF-Y complex binding to DNA are located within the NF-YA subunit (Xing et al., 1993) and these residues are not shared by CO (Siefers et al., 2009).

It was recently reported that bZIP67, LEAFY COTYLEDON 1 (LEC1, NF-YB9), LEC1-LIKE (NF-YB6), and NF-YC2 can interact to promote the expression of two abcisic acid responsive promoters (Yamamoto et al., 2009b). Consistent with the idea that NF-YA proteins can act as negative regulatory factors, inclusion of NF-YA4, 5, 7, and 9 in the same reporter assays interfered with gene activation. Thus, NF-Y complexes that extend beyond the canonical NF-YA/B/C trimer established in animal systems may be commonplace in the plant lineage.

An alternative hypothesis to antagonism between CO and NF-YA is that CO interacts with the entire NF-Y heterotrimer (NF-YA subunit included) and functions as an activation domain. In our Y2H experiments, we found that full length CO was a very strong transcriptional activator when bound to a DNA binding domain. CO interaction with complete NF-Y complexes would be analogous to yeast where an orthologous NF-Y trimer (HAP2, HAP3 and HAP5) binds DNA while an additional subunit, called HAP4, provides the transcriptional activation domain (Forsburg and Guarente, 1989). If this scenario were correct, one would predict that loss of function mutants in appropriate *NF-YA* genes would show late flowering phenotypes. Due to high sequence similarity between Arabidopsis NF-YA proteins, higher order mutants will also likely be necessary to test this scenario and elucidate their function in flowering (Li et al., 2008b; Siefers et al., 2009).

NF-Y contribute to many aspects of plant growth and development, but no complete NF-Y complex has been identified for a specific process. Because of the high level of similarity and evolutionary conservation between NF-Y proteins in human and plants, we assume that they typically function in an analogous manner. Nevertheless, due to the numerical expansion of *NF-Y* in the plant lineage, many interesting functional nuances are possible. In our opinion, describing NF-YA functions is currently a particularly important area for understanding these transcription factors. Now that NF-YB and NF-YC subunits required for CO function in photoperiod-dependent flowering have been discovered, we can proceed to this next step with increased confidence.

Materials and Methods

Phylogenetic Analysis

The phylogenetic tree shown in Figure 2.1 was generated using the publicly available software package MEGA 4 (Tamura et al., 2007). This tree was adapted from Siefers et al. (2009) and is included again here for ease of visualizing NF-YC relationships.

Plant Cultivation, GUS Staining, and Flowering Time Experiments

All plants were Col-0 ecotype and were grown at 23C in standard LD (16h light/8 hr dark) and SD (8h light/16 hr dark) conditions in Conviron (www.conviron.com) Model ATC13 growth chambers or a custom walk-in chamber. Plants were grown in media containing equal parts Farfard C2 Mix

and Metromix 200 supplemented with 40 grams Marathon pesticide and dilute Peters fertilizer (NPK = 20:20:20). Plants were watered throughout with dilute fertilizer (Peters 20:20:20/N:P:K at ~1/10 recommended feeding levels).

GUS staining was done on the first set of true leaves of 10 day old plants as previously described (Malamy and Benfey, 1997). Leaf images were visualized on an Olympus BX41 microscope (www.olympusamerica.com) and recorded with a Diaginic Insight 2 Megapixel Color Mosiac CCD camera and SPOT software (version 4.6, www.diaginc.com).

Leaf number at flowering was determined by counting all primary rosette leaves and cauline leaves just after the first flowers opened (Onouchi et al., 2000). None of the mutant lines exhibited enhanced developmental rates.

DNA Manipulations and Transgenic Plants

Unless otherwise noted, all clones were generated by PCR using Pfu Ultra II (Stratagene cat#600670-51, www.stratagene.com) and ligation into the GATEWAY™ entry vector pENTR/D-TOPO (Invitrogen cat#45-0218, www.invitrogen.com). Full length (minus the stop codon) *NF-YB2*, *NF-YB3*, *NF-YC3*, *NF-YC4*, and *NF-YC9* were derived from Col-0 cDNA populations by standard methods (cloning primers available upon request). The *CO* cDNA construct used in Y2H analyses was obtained from a pENTR223 construct available at ABRC (stock#GC105432). Full length *CO,* used for *in planta*

overexpression, was amplified from genomic DNA and includes introns. The complementation constructs for *NF-YC3*, *4* and *9* contain the genomic region corresponding to -1,000 from the ATG through the genomic coding region (minus the stop codon). All constructs were sequenced and found to be identical to the expected sequences found at The Arabidopsis Information Resource (Swarbreck et al., 2008). Subsequent clones into plant expression and Y2H vectors were created using the GATEWAY™ LR Clonease II reaction kit (Invitrogen cat#56485). Plant expression complementation constructs were cloned into pEarleyGate301 (ABRC stock#CD3-692, (Earley et al., 2006)). Epitope tagged, over-expression constructs of *NF-YB2*, *NF-YB3*, and *CO* were cloned into pEarleyGate101 (ABRC Stock #CD3-683, (Earley et al., 2006)). Promoter GUS fusion lines were previously described (Siefers et al., 2009). All primer sequences are available upon request. All plant transformations were done by the Agrobacterium-mediated, floral dipping method as previously described (Bechtold et al., 1993; Clough and Bent, 1998).

qRT-PCR Analyses

Total RNA was isolated from 10 day old seedlings grown under LD conditions using the Qiagen RNeasy Plant Mini Kit (cat#74904, www1.qiagen.com). Samples were collected either over a diurnal time course or 15 hours after lights on. Isolated RNA was DNase treated (Ambion cat#AM2222, www.ambion.com) and first strand cDNA synthesis was performed using the Ambion RETROscript kit (cat#1710) with supplied oligo dT primers. qRT-PCR was performed as

previously described, except we used an Applied Biosystems (www.AppliedBiosystems.com) Prism 7500 analyzer and the Fermentas Maxima SYBR Green qPCR Master Mix (cat#K0222, www.fermentas.com, (Kumimoto et al., 2008). For each genotype, we analyzed 3-4 independent, biological replicates. 10 plants were combined for each biological replicate. All samples were normalized to the constitutively expressed gene *At2g32170* as previously described (Czechowski et al., 2005). Sample comparisons were performed by the 2(-Delta Delta C(T)) method (Livak and Schmittgen, 2001) and errors (standard deviation) were computed as previously described (Nordgard et al., 2006).

Microarray Analysis

All microarray data was collected and recorded in compliance with MIAME (Brazma et al., 2001). Unprocessed microarray data (.cel and .chp files) and detailed experimental conditions are publicly available at the NASCarrays website (http://affymetrix.arabidopsis.info/narrays/experimentpage.pl?experimentid=440) using the experiment identifier NASCARRAYS-440. Briefly, plants for microarray analysis were grown for 10 days under standard long day conditions; above ground tissue was harvested 14 hours after chamber lights on. Plant density and spacing were equivalent for all biological replicates performed. For each plant genotype - Col-0 parental control, *nf-yc triple*, and *co-sail* - total RNA was collected from 10 plants/biological replicate using the

Qiagen RNeasy Plant Mini Kit. Total RNA from three biological replicates was collected for a total of 9 independent RNA preparations and microarray hybridizations. Isolated total RNA was sent to the Nottingham Arabidopsis Stock Center (NASC) for quality control and hybridization to Affymetrix ATH-1 microarray chips (www.affymetrix.com). Resulting data were analyzed with the GeneSpringGX 7.3.1 software package (Agilent Technologies, www.agilent.com). Data were GCRMA normalized to the 50th percentile during data importation into GeneSpring. Variances were calculated using the cross gene error model with replicates. With cross gene error model active, we performed ANOVA with Benjamini Hochberg, false discovery rate corrected P values ($p<0.05$). Significantly misregulated genes, as determined by ANOVA, were further filtered by volcano plot analysis ($p<0.05$, fold change >1.5) in pairwise comparisons between the parental control and each mutant genotype.

Protein Blots, Antibodies, and Co-Immunoprecipitation

In Figure 2.1D, total protein was extracted from 10-12 day old seedlings by grinding fresh tissue in sucrose buffer - 20mM Tris, pH 8.0, 0.33M sucrose, 1mM EDTA, pH 8.0; added fresh 5mM DTT and 1X Sigma Protease Inhibitor Cocktail (cat# P9599, www.sigmaaldrich.com). Proteins were visualized by standard SDS-PAGE methods (15% gel). Western blotting was also done by standard methods (as described in the ECL Plus Reagent protocol, GE Healthcare/Amersham cat# RPN2132, www.gelifesciences.com). Briefly, we used PVDF membranes (Hybond-P, GE Healthcare/Amersham Biosciences,

cat# RPN2020F) and blocked with tris buffered saline containing 0.05% Trition X-100 (TBS-T) plus 5% skim milk. NF-YC3 and NF-YC4 primary antibodies (described below) in TBS-T plus 1% skim milk were followed by hybridization to goat anti-IG-Y secondary antibodies (Santa Cruz Biotechnology, cat # SC-2428, www.scbt.com). After each antibody incubation (2 hours primary, 1 hour secondary), blots were washed three times (15 minutes each) with excess TBS-T. Protein blots were visualized using the horseradish-peroxidase based ECL Plus reagent on a GE Storm 960 Phosphorimager.

IG-Y antibodies specific for NF-YC3 and NF-YC4 were generated by injecting chickens with pairs of protein-specific synthetic peptide antigens. Peptide design and the production of initial, non-affinity purified IG-Y antibodies was provided as a service by Aves Labs (Tigard, OR, www.aveslab.com). For NF-YC3, chickens were injected with the synthetic peptides CZTTTPTGSDHPAYHQIHQQ and CZQQPGPEQQDPDN. For NF-YC4, chickens were injected with the synthetic peptides CZEEIKEEEDAASA and CZTSVYPPGSAVTTV.

IG-Y antibodies from Aves Labs were purified with the Thermo Scientific/Pierce SulfoLink Immobilization Kit for Peptides (cat#44999, www.piercenet.com) as described by the manufacturer. Antibodies were eluted from the columns by 0.2M glycine-HCL (pH 2.5) directly into 1.0M Tris-HCL (pH 8.0). Equal volumes of 100% saturated, cold ammonium sulfate (pH 7.2) were added and antibodies

were allowed to precipitate overnight at 4C. The resulting precipitates were concentrated by centrifugation (3,500 rpm for 30 minutes) and resuspended in 1X PBS. Resuspended antibodies were dialyzed against large volumes of cold PBS for ~24 hours with three buffer changes.

NF-YC3 and NF-YC4 antibodies detect their targets at 1:5,000-10,000 and 1:2,000 dilutions, respectively. Goat anti IG-Y was used at 1:5,000 in all experiments. NF-YC4 antibodies require longer incubations for effective signal visualization (2 hrs at room temperature or overnight at 4C). We will accommodate requests for samples of these affinity purified antibodies, but we reserve the right to limit their future distribution such that we retain sufficient working stocks.

For the co-immunoprecipitation in Figure 5, we used hepes lysis buffer (50mM hepes, pH 7.5; 150mM NaCl; 0.5% NP-40; 10% glycerol; add fresh 1mM PMSF, 5µg/µl aproptinin, and 5µg/µl pepstatin) to extract total proteins. For each sample, 1mg of total protein was brought to a final volume of 500µl in a 1.5 ml Eppendorf tube and 5µl of anti-HA antibody was added (ABCAM cat#AB9110, www.abcam.com). Proteins and antibodies were incubated on a rotator at 4C for 4 hours. Pre-equilibrated (with hepes lysis buffer) Sepharose A agarose beads (70µl bead volume in ~100µl) were then incubated with the protein/antibody mixture for 4 hours. Sepharose A agarose beads were washed by three rounds of full speed centrifugation at 4C, supernatant removal, and

resuspension in 1ml wash buffer (hepes buffer above, except 50mM NaCl, 0.1% NP-40, and no glycerol). The final pellet was incubated with 100µl of standard 2X SDS sample buffer for 5 minutes at 90C and then stored at -80C until western analysis (as described above). NF-YB/NF-YC co-immunoprecipitation was repeated three times with similar results.

Yeast Two-Hybrid Analysis

Gateway™ entry clones (above) were transferred into the ProQuest Two-Hybrid System (Invitrogen) vectors pDEST22 and pDEST32. All interaction tests between full length proteins were performed exactly as described in the ProQuest manual. The Y2H library we screened was an equal parts mixture of four previously described, Gateway™-ready libraries made from both hormone treated and untreated seedlings, flowers, developing seeds, and primary leaves (Burkle et al., 2005).

Figures and Tables

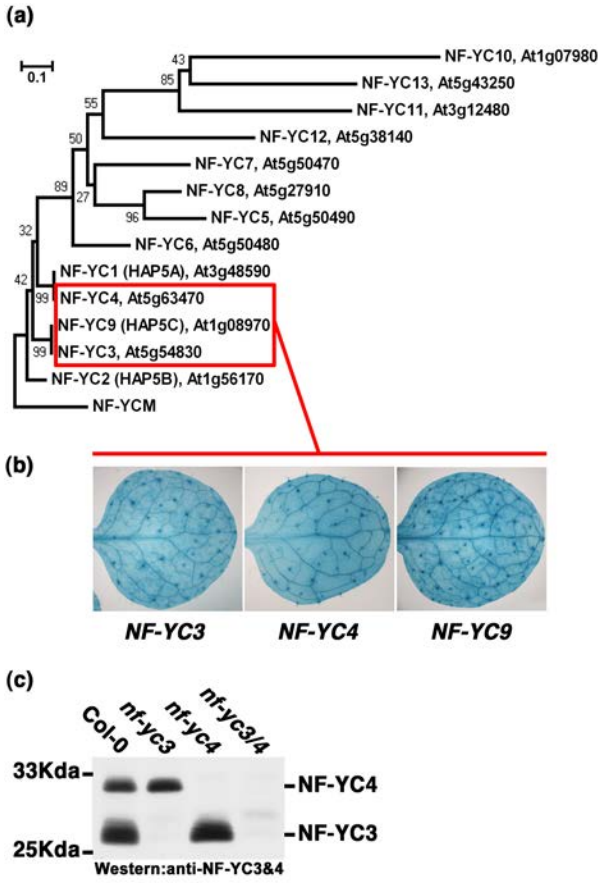


Figure 2.1. A subset of *NF-YC* have consistently strong vascular expression. A) Phylogenetic relationships of 13 Arabidopsis *NF-YC*. Vascular-expressed genes highlighted by red box. Panel adapted from Siefers et al. (2009). **B)** Promoter:GUS expression patterns for vascular-expressed *NF-YC*. Only *NF-YC3*, *4*, and *9* had strong and consistent vascular expression in 10 day old plants. **C)** Protein blot analysis using native IG-Y antibodies raised against peptides specific for NF-YC3 and NF-YC4.

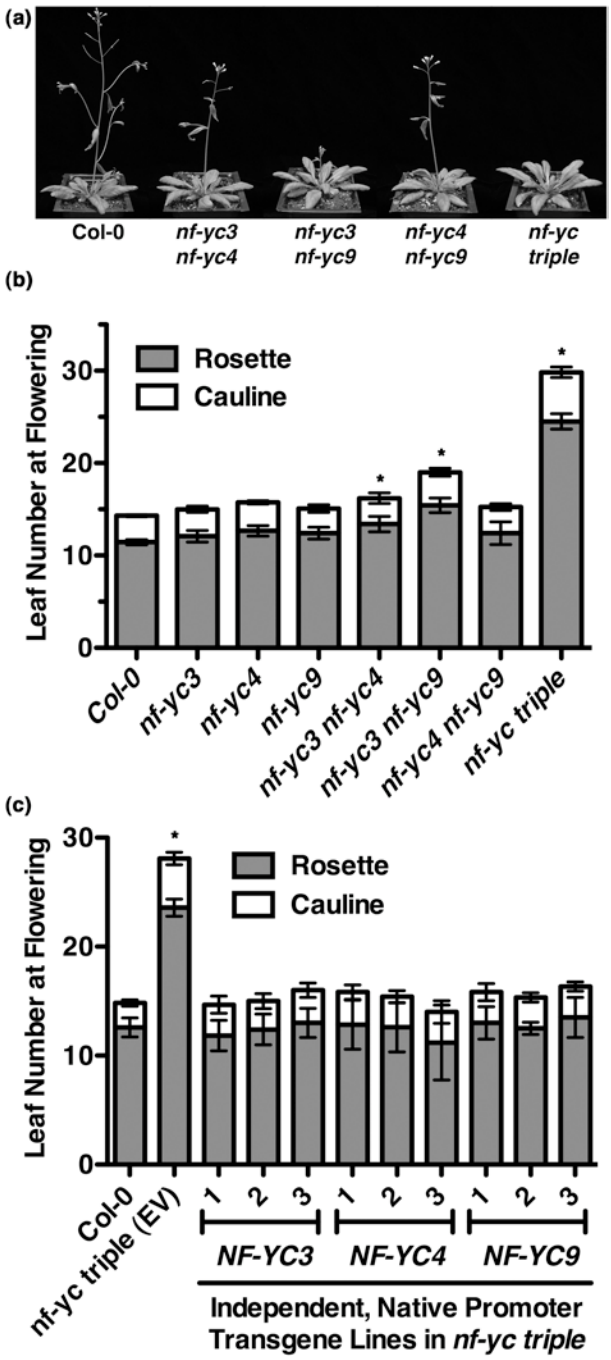


Figure 2.2. *nf-yc* mutants are delayed in flowering under LD conditions. A) Mutant plant phenotypes (day 32) compared to parental Col-0. **B)** Comparison of leaf number at flowering for all possible *nf-yc3*, *nf-yc4*, and *nf-yc9* mutant combinations. **C)** Complementation of the *nf-yc triple* mutant by *NF-YC3*, *4*, and *9* genomic constructs in LD conditions. Three independent, basta-selected T2 populations were examined for each genomic construct. Bars in all panels represent means for *at least* 10 plants/genetic background (±95% CI) and asterisks represent significantly different comparisons to the Col-0 parental ecotype (ANOVA $p<0.0001$, Bonferroni multiple comparison test (BMCP), $p<0.001$).

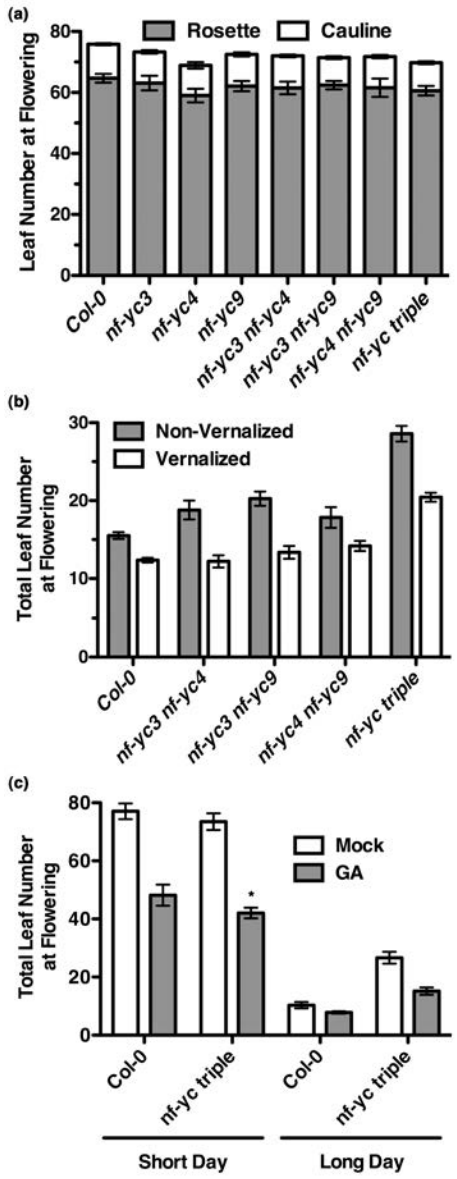


Figure 2.3. Alternative flowering pathways are largely unaffected in the *nf-yc triple*.

Mean leaf number at flowering under **A)** SD conditions (8hrs light/16 hrs dark), **B)** vernalization treatment (10 weeks at 4C, moist growth media, ~100% humidity), and **C)** GA_3 treatment (SD and LD, see text). Asterisks represent significantly different comparisons to the appropriate Col-0 control (ANOVA, $p<0.0001$, BMCP, $p<0.001$). Error bars ±95% CI.

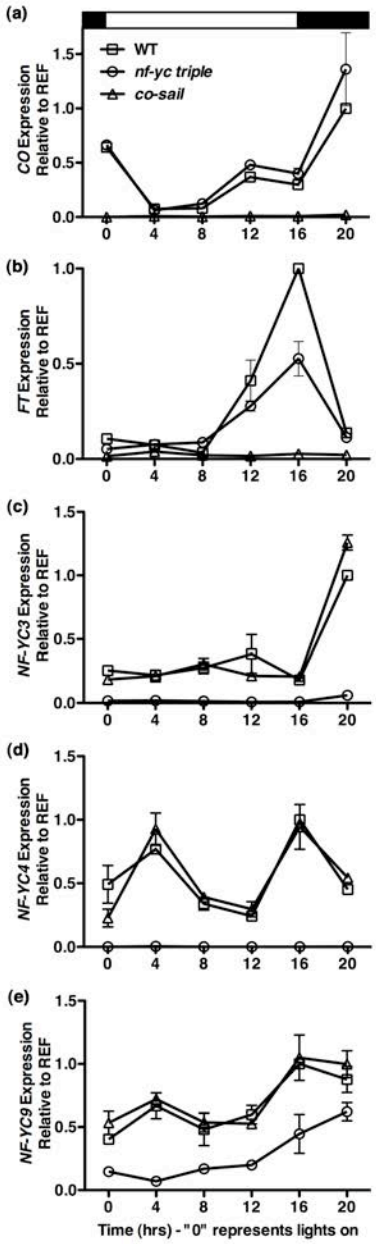


Figure 2.4. Relative expression levels of *CO*, *FT*, *NF-YC3*, *4*, and *9* in flowering time mutants by qRT-PCR over a time course. A) *CO*, **B)** *FT*, **C)** *NF-YC3*, **D)** *NF-YC4*, and **E)** *NF-YC9* expression in *nf-yc triple*, *co-sail*, and *35S:CO* genetic backgrounds. Expression levels presented relative to reference gene (±SE, see Methods).

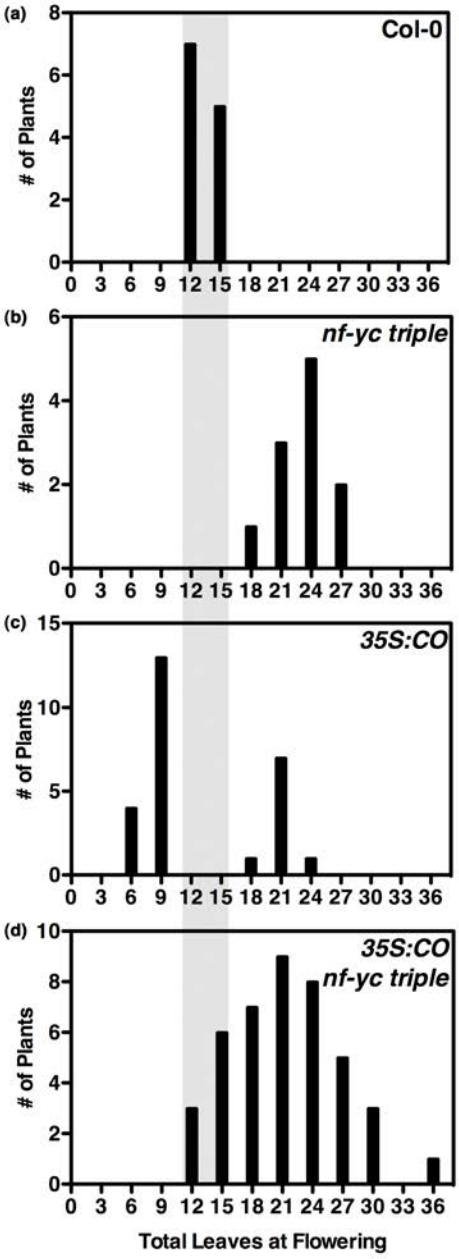


Figure 2.5. *CO* requires *NF-YC 3*, *4*, and *9* to drive early flowering. Data represents plant frequency distributions based on total leaf number at flowering (bins = 3 leaves; i.e., "12" = range of 11 to 13). **A)** Col-0 (n=12), **B)** *nf-yc triple* (n=11), **C)** *35S:CO* (n=26 T1 individuals), and **D)** *35S:CO nf-yc triple* (n=42 T1 individuals). Grey bar extending through each panel represents the range of flowering time responses in Col-0.

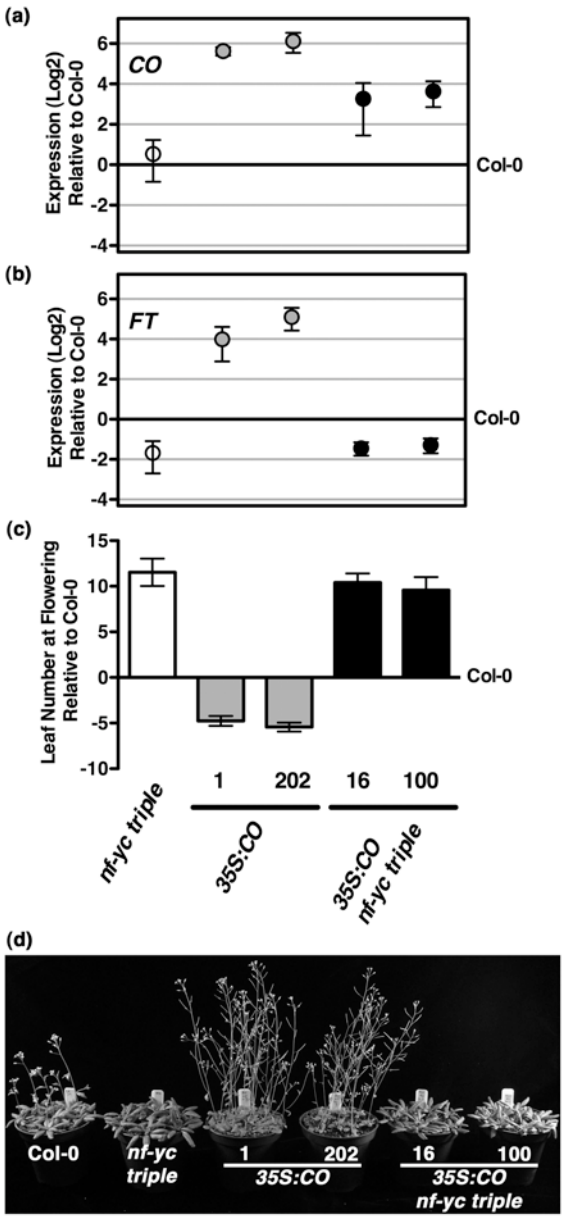


Figure 2.6. *35S:CO* does not significantly alter *FT* expression in the *nf-yc triple* background. A-C) "0" line represents the Col-0 mean. **A)** Mean *CO* and **B)** mean *FT* mRNA expression measured by qRT-PCR (Log2 values, ±SD). **C-D)** Mean (±95% CI) total leaf number at flowering for stable, homozygous T3 lines relative to Col-0 and pictures of representative plants for each genotype. Col-0 flowered at 12.8 leaves in this experiment.

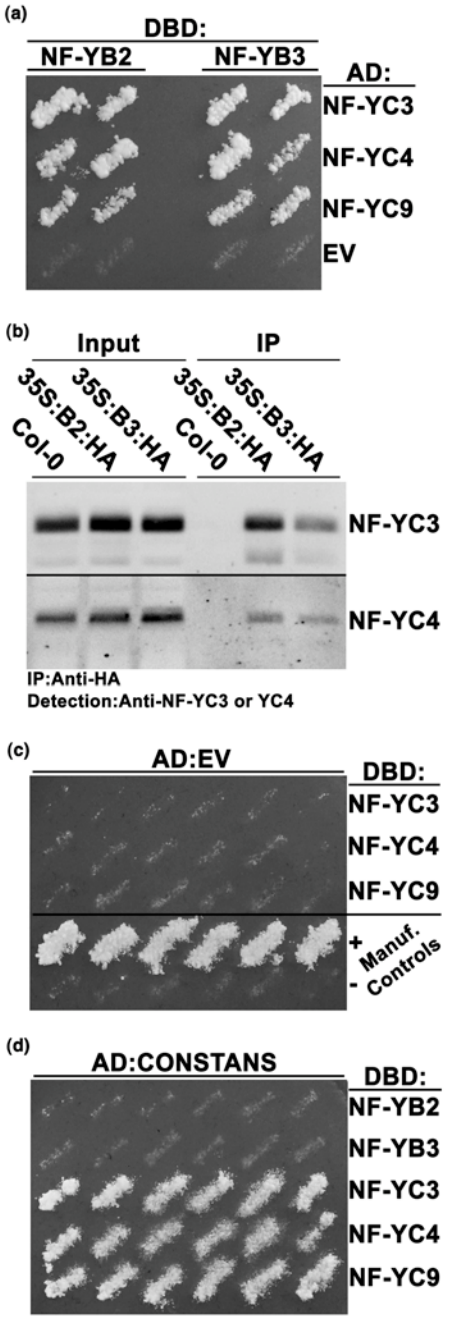
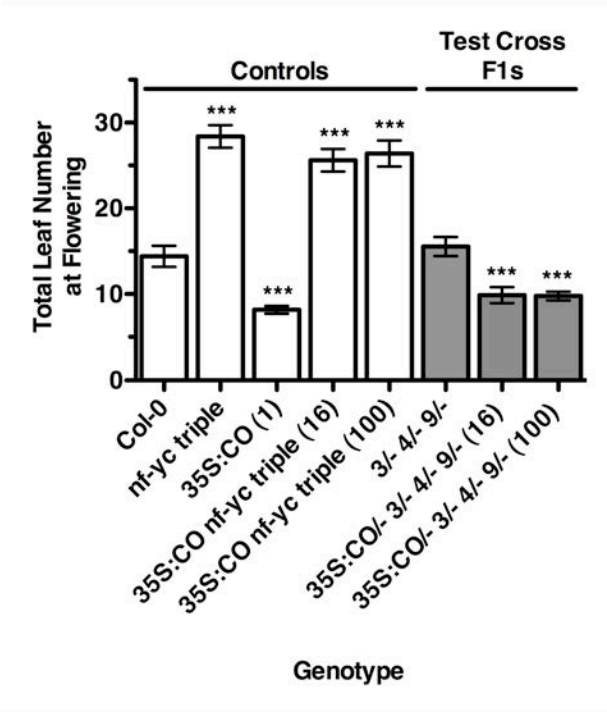
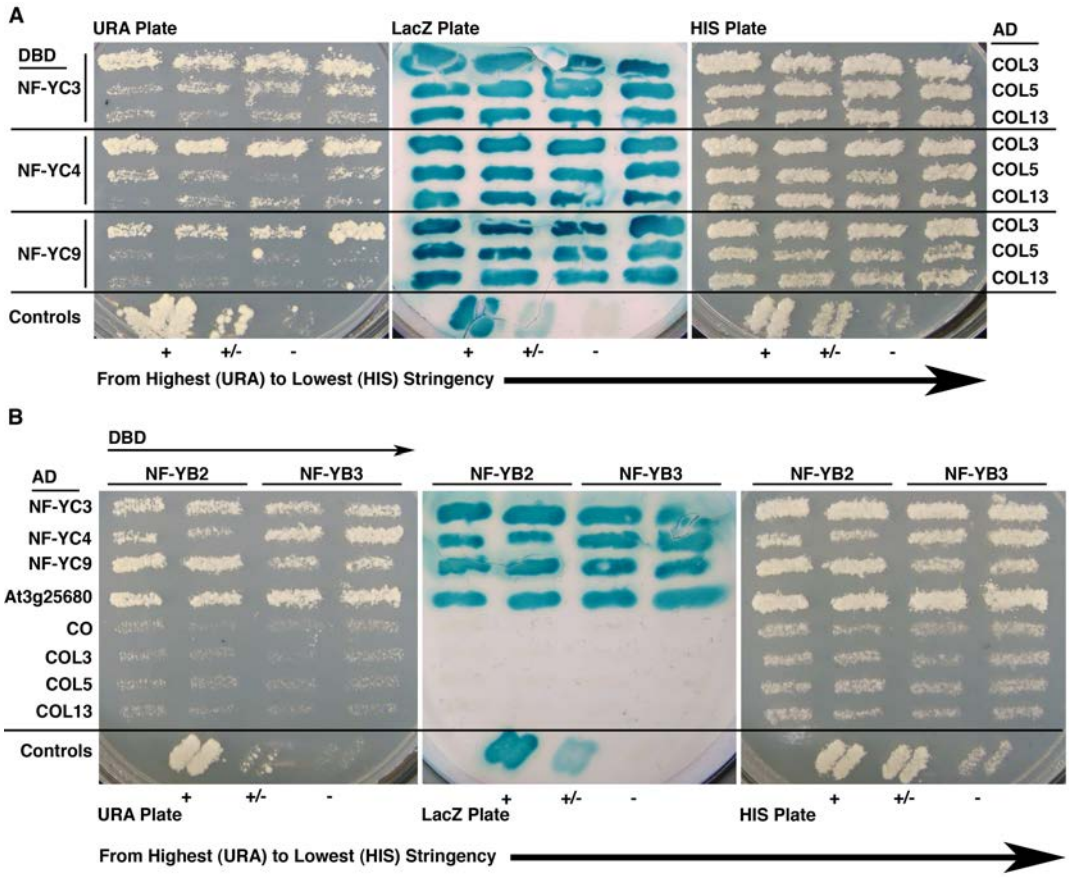


Figure 2.7. NF-YC3, 4, and 9 physically interact with NF-YB2, 3 and CO. A) Y2H analysis of interactions between Gal4 DNA binding domain (DBD):NF-YB2, 3 and Gal4 activation domain (AD):NF-YC3,4, 9 fusions - yeast growth shown on uracil deficient plates and additionally confirmed with both His and LacZ reporter genes. EV - empty vector control shows that neither NF-YB2 or 3 autoactivate the reporter gene. **B)** Protein blot of *in vivo* interaction between NF-YB2, 3 and NF-YC3, 4. **C)** Y2H control plate for DBD:NF-YC3, 4, 9 constructs - none autoactivate reporter. + and - lines represent manufacturer's controls (ProQuest, Invitrogen). **D)** Y2H interaction tests between NF-YB2, 3, NF-YC3, 4, and 9 against full length CO - six independent colonies tested/interaction.



Supplemental Figure 2.1. ***35S:CO-YFP/HA*** **Lines 16 and 100 can drive early flowering in the context of functional NF-YC3, 4, and 9.** Numbers in parentheticals after genotypes represent the line numbers described in the main text and Figure 6. 35S:CO/- 3/- 4/- 9/- is shorthand for hemizygous *35S:CO-YFP/HA* transgene in the *NF-YC3/nf-yc3-1 NF-YC4/nf-yc4-1 NF-YC9/nf-yc9-1* triple heterozygous background. Prior to statistical analysis, data were transformed (1/(Y)) to correct for unequal standard deviations between groups. *** represents significant differences ($p<0.001$) in pairwise comparisons to Col-0 (Bonferroni multiple comparison test) after significant ANOVA ($p<0.0001$).



Supplemental Figure 2.2. NF-YB2 and 3 physically interact with NF-YC3, 4, 9, and At3g25680 by Y2H, but not with full length CO and COL proteins. A) NF-YC3, 4, and 9 interactions with various full length COL proteins. AD - Activation Domain fusion; DBD - DNA binding domain fusion. Based on our experiences, reporter gene stringencies are aligned from highest (URA) to lowest (HIS). For example, note that NF-YC interactions are generally weaker with COL5 and COL13 as evidenced by the URA plate. +, +/-, and - yeast streaks represent the manufacturer's (Invitrogen) provided controls and should be compared to the experimental interactions on a plate by plate basis. **B)** NF-YB2 and 3 interaction tests with CO and COL proteins. NF-YC3, 4, 9, and At3g25680 represent known positive interaction controls (At3g25680 is a protein of unknown function and was the most abundant non-NF-YC interactor in our library screen with 19/51 (37%) sequenced clones).

Chapter 3: NF-Y have both opposing and additive roles in ABA-mediated seed germination

This Chapter has been reviewed for publication at PloS ONE. All requested revisions were made and it will be returned to PloS ONE in December 2012.

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Abstract

In the model organism *Arabidopsis thaliana* the heterotrimeric transcription factor NUCLEAR FACTOR Y (NF-Y) has been shown to play multiple roles in facilitating plant growth and development**.** Although NF-Y itself represents a multi-protein transcriptional complex, recent studies have shown important interactions with other transcription factors, especially those in the bZIP family. Here we add to the growing evidence that NF-Y and bZIP form common complexes to affect many processes. We carried out transcriptional profiling on *nf-yc* mutants and through subsequent analyses found an enrichment of bZIP binding sites in the promoter elements of misregulated genes. Using NF-Y as bait, yeast two hybrid assays yielded interactions with bZIP proteins that are known to control ABA signaling. Accordingly, we find that plants mutant for several NF-Y subunits show characteristic phenotypes associated with the disruption of ABA signaling. While previous reports have shown additive roles for *NF-YC* family members in photoperiodic flowering, we found that they can have opposing roles in ABA signaling. Collectively, these results demonstrated the importance and complexity of *NF-Y* in the integration of environmental and hormone signals.

Introduction

Successful acclimation of plants to temporal environmental changes requires the integration of multiple intersecting signals. These environmental signals, such as changing light and water availability, profoundly modify growth and developmental programs. Phytohormones often play a central role in these responses and recent studies identified several integrators of light and hormone signaling pathways (reviewed in (Lau and Deng, 2010) and (Jaillais and Chory, 2010)). For example, the basic leucine zipper (bZIP) transcription factor LONG HYPOCOTYL 5 (HY5) has been intensely studied for its roles in light regulated development, but was only recently found to additionally mediate abscisic acid (ABA) signaling in germinating seeds (Chen et al., 2008). Thus, pathway integrators can act as hubs for multiple environmental inputs, but how they coordinate these variable inputs to generate unique transcriptional outputs remains unknown (Jaillais and Chory, 2010).

One possibility for coordinating multiple environmental inputs is through interactions with combinatorial transcription factors. Increasing evidence suggests that the combinatorial transcription factor NUCLEAR FACTOR Y (NF-Y) plays important roles in facilitating plant responses to various environmental signals and hormones (Ben-Naim et al., 2006; Wenkel et al., 2006; Warpeha et al., 2007; Yamamoto et al., 2009a). In fact, like HY5, NF-Y complexes can regulate both light signaling (blue light) and hormone perception (ABA, (Warpeha et al., 2007)). NF-Y transcription factors bind at *CCAAT cis*-elements

and function as trimeric complexes consisting of three distinct protein subunits, NF-YA (NF-Y, subunit A), NF-YB, and NF-YC (Maity and de Crombrugghe, 1998). These individual subunits are each encoded by small gene families found throughout the plant lineage – e.g., *Arabidopsis thaliana* (Arabidopsis) has 36 *NF-Y* encoding genes (10 *NF-YA*, 13 *NF-YB*, and 13 *NF-YC* (Siefers et al., 2009)). Because the mature DNA-binding NF-Y complex is thought to contain only one of each subunit type, hundreds of unique combinations are theoretically possible. Thus, unique combinations of NF-Y transcription factors may provide a flexible system for fine-tuning the integration of environmental signals to transcriptional outputs (Siefers et al., 2009; Cao et al., 2011; Li et al., 2011).

NF-Y are known to regulate a variety of developmental phenotypes and stress responses. For example, NF-YB and NF-YC subunits regulate photoperiod-dependent flowering (Ben-Naim et al., 2006; Wenkel et al., 2006; Cai et al., 2007; Kumimoto et al., 2008; Kumimoto et al., 2010; Li et al., 2011) and overexpression of both NF-YA and NF-YB proteins can confer drought tolerance in plants (Nelson et al., 2007; Li et al., 2008a). In the legumes *Phaseolus vulgaris* and *Medicago truncatula*, specific NF-YA and NF-YC subunits are necessary for the development of nitrogen fixing nodules (Combier et al., 2006; Combier et al., 2008; Zanetti et al., 2011). Other studies have implicated NF-Ys in embryogenesis, light perception, unfolded protein responses (UPR), and photosynthesis (Meinke et al., 1994; West et al., 1994;

Masiero et al., 2002; Lee et al., 2003; Warpeha et al., 2007; Liu and Howell, 2010; Stephenson et al., 2010).

Although NF-Y itself represents a multi-protein complex that can independently integrate multiple signals, recent studies have shown additional interactions with other transcription factor families, especially those in the bZIP family. Yamamoto *et al.* (2009a) demonstrated that central regulators of embryogenesis, LEAFY COTYLEDON 1 (LEC1/NF-YB9) and LEC1-LIKE (L1L/NF-YB6), interact with bZIP67 to regulate the expression of genes with ABA responsive elements (ABRE) in their promoters. More recently, Liu and Howell (2010) proposed a model where a complex of bZIP28, NF-YA4, NF-YB3, and NF-YC2 binds to endoplasmic reticulum stress response elements (ERSE) to regulate genes related to the unfolded protein response (UPR). In both cases, full activation of target promoters required the presence of NF-Y and bZIP components. Therefore, the complexity of NF-Y transcription factors can be further increased through interactions with bZIP proteins.

Like NF-Ys, bZIPs are also found throughout the eukaryotic lineage. In Arabidopsis bZIP proteins represent a large family containing 75 members in ~20 phylogenetically related sub-groups (Jakoby et al., 2002). Similar to NF-Y, bZIP proteins are involved in diverse processes ranging from environmental stress tolerance to development. Group A bZIP proteins are particularly important for the integration of environmental stress signals (e.g., drought) with

hormone-related responses (especially ABA, (Fujita et al., 2011)). Examples of Group A bZIP proteins include the previously discussed bZIP67, as well as ABSCISIC ACID INSENSITIVE 5 (ABI5) and the ABA RESPONSE ELEMENT BINDING PROTEINS/ABA BINDING FACTORS 1-4 (AREB/ABF1-4). Mutations in *ABI5* allow developing seeds to germinate and grow on normally restrictive levels of ABA (Finkelstein, 1994). Mutant and overexpression lines for *ABF1-4* show characteristic morphological and molecular phenotypes typically associated with ABA responses, including altered growth, transpiration rates, and seed germination in response to abiotic stress (Umezawa et al., 2010). Although not in Group A, the light and ABA-signaling integrator HY5 is also a bZIP protein. HY5 directly regulates the expression of *ABI5* and *hy5* mutants have reduced sensitivity to ABA in a variety of assays, including seed germination and root growth (Chen et al., 2008).

Previously, we showed that *NF-YC3*, *NF-YC4*, and *NF-YC9* have overlapping roles in photoperiod-dependent flowering (Kumimoto et al., 2010). In the current study we used transcription profile analysis of *nf-yc3 nf-yc4 nf-yc9* triple mutants to investigate other pathways potentially affected by these genes. This analysis revealed an over-representation of *cis*-elements with putative bZIP binding sites. We hypothesized that *NF-Y* mutants would share phenotypic similarities to various *bZIP* mutants and tested this idea by assaying seed germination, seedling greening, and root elongation for abnormalities as well as quantitative reverse transcriptase polymerase chain reaction analyses (qPCR)

on known bZIP regulated genes. We found that various mutant and overexpression combinations of *NF-YB* and *NF-YC* genes significantly altered characteristic morphological and molecular phenotypes associated with ABA signaling. Additionally, we found that NF-Y subunits from the same family can sometimes have opposing roles in a given process. Overall, these data suggested that NF-Y and bZIP transcription factors coordinately regulate ABA-related phenotypes.

Results

Misregulated genes in *nf-yc* mutants are enriched for bZIP binding sites

We previously used qPCR and microarray analyses to demonstrate that *FLOWERING LOCUS T* (*FT*) was downregulated in the 10 day-old seedlings of long day grown *nf-yc3 nf-yc4 nf-yc9* triple mutants (hereafter *nf-yc triple*) (Kumimoto et al., 2010). To generate new hypotheses and extend the *nf-yc triple* analysis here, we further examined this published microarray data by searching for over-represented promoter motifs within the 5' upstream sequences of the misregulated gene set (both up and down regulated genes, $p<0.05$ without false discovery rate correction; see Materials and Methods). This set consisted of 83 nuclear-encoded genes that were at least 1.5 fold misregulated in the *nf-yc triple*. Initially, we used the online software package Athena to search this gene set for enriched promoter motifs (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl, (O'Connor et al., 2005)).

Using the Athena analysis suite, three statistically over-represented promoter motifs were discovered (Table 3.1). All three were bZIP-binding sites with the core conserved sequence *ACGT*. The most highly significant motif was the 3.3-fold over-represented *CACGTG* motif, also called the G-box (Giuliano et al., 1988). The G-box is found in the promoters of many light responsive genes and is a known binding site for HY5 (Koornneef et al., 1980; Ang et al., 1998a; Lee et al., 2007b). The other two significantly enriched sequences were related to the ABRE and also have the *ACGTG* portion of the G-box. ABREs are also bound by bZIP proteins, specifically the Group A bZIP transcription factors AREB/ABF1-4 (Choi et al., 2000; Uno et al., 2000; Jakoby et al., 2002). These data are consistent with a previous report that L1L (NF-YB6) (Kwong et al., 2003; Lee et al., 2003) physically interacts with the Group A bZIP protein bZIP67 to regulate ABRE containing promoters (Yamamoto et al., 2009a). Also of note in Table 3.1 is the enrichment of the unfolded protein response (UPR) element, as identified in animal systems (Yoshida et al., 1998). Although just missing statistical significance in our dataset, Liu and Howell recently showed that NF-Ys can physically interact with bZIP28 and affect the expression of genes with the UPR promoter element (Liu and Howell, 2010).

In addition to Athena, we used MEME (Multiple Em for Motif Elicitation, http://meme.nbcr.net) to search for shared motifs in the misregulated gene set (Bailey and Elkan, 1994; Bailey et al., 2006). MEME uses a Hidden-Markov

model and is not constrained by searching for known motifs (i.e., it can find both known and novel motifs). Three low E-value motifs were discovered and each was tested for resemblance to previously identified transcription factor binding sites using the TOMTOM motif comparison tool (Gupta et al., 2007). While two of the motifs showed no clear homology to known binding sites, one showed significant resemblance to known bZIP binding sites, including those bound by ABF, HY5 and EmBP-1 (Figure 3.1A). Consistent with our previous results, bZIP binding sites were often located between -100 to -200bp of the transcription start site within the promoters of the misregulated gene set (Figure 3.1B). These data, as well as several recent reports (Yamamoto et al., 2009a; Liu and Howell, 2010), suggest that NF-Y may have a generalizable relationship with bZIP proteins in the transcriptional regulation of numerous genes.

Predicted NF-YC interaction network include bZIP proteins

Bioinformatic analysis of promoter elements enriched on the *nf-yc triple* microarray revealed a possible interaction with bZIP domain containing proteins. To further investigate the predicted protein-protein interaction network of NF-YC3, NF-YC4, and NF-YC9, we used the web based software GeneMANIA which is designed to identify functional associations (Mostafavi et al., 2008; Warde-Farley et al., 2010). For visualization, individual protein-protein interaction networks from GeneMANIA were integrated into a single network map using the open source software Cytoscape (Figure 3.2, (Cline et al., 2007)). In addition to the predicted interaction network provided by

GeneMANIA, we included previously published NF-Y by CCT (Constans, Constans-Like, TOC1) protein interactions. We also included bZIP and CCT protein interactions discovered in a yeast two hybrid (Y2H) screen using NF-YC9 as bait and the directed Y2H data shown below ((Kumimoto et al., 2010) and RWK and BFH unpublished). As expected, all 13 Arabidopsis NF-YB proteins, as well as four of 10 NF-YA proteins, were predicted to interact with the NF-YC proteins. In addition to the previously described interactions with NF-YA, NF-YB and CCT proteins (Ben-Naim et al., 2006; Wenkel et al., 2006; Kumimoto et al., 2010), there were two other large classes of proteins represented on the network map; histone 2A and bZIP containing proteins. These data further support the bZIP binding site enrichment found in the *nf-yc triple* microarray gene set, as well as previously reported interactions between NF-Y and bZIP proteins.

NF-Y can physically interact with ABA related bZIP proteins.

Relevant to the promoter analyses, NF-YC interaction network, and hypothesis that bZIP/NF-Y interactions are common, we isolated bZIP proteins in Y2H screens using both NF-YB2 and NF-YC9 as bait. When NF-YB2 was used as the bait, we isolated and sequenced 42 positive clones. Five of the positive clones were either bZIP1 (2 independent clones) or ABF3 (3 independent clones). Further, a screen using NF-YC9 as bait yielded a single clone of ABF2 out of 200 sequenced positives. Although a single clone of ABF2 appears low, it

should be noted that 78% of the sequenced positive clones from the NF-YC9 screen were either NF-YB or CCT proteins (155 out of 200).

To further validate and extend the bZIP/NF-Y interactions from the Y2H library screens, we independently cloned full length versions of five bZIP genes with known roles in ABA responses: ABF1, ABF2, ABF3, ABF4 and HY5. Each of these was tested for interaction with a panel of NF-YB and NF-YC proteins in directed Y2H assays. For the NF-YC proteins, we chose the members of the *nf-yc triple* used in the above microarray analysis (NF-YC3, NF-YC4, and NF-YC9). These three proteins are known to genetically interact in an overlapping manner to control *FT* expression and presumably they are interchangeable in the NF-Y complexes controlling *FT* transcription (Kumimoto et al., 2010). Likewise, we tested NF-YB2 and NF-YB3 against the panel of bZIPs. NF-YB2 and NF-YB3 are known *in vivo* interactors with NF-YC3 and NF-YC4 and also have overlapping functions in the control of *FT* expression (Kumimoto et al., 2008; Kumimoto et al., 2010). Finally, NF-YB1 was tested because of its known roles in drought resistance (Nelson et al., 2007) - i.e., we hypothesized a possible connection to the known roles of the ABF/AREBs in various ABA-related responses, including drought resistance.

NF-YC4 and NF-YC9 interacted with all five of the tested bZIP proteins (3.3A). NF-YC3 also likely interacted with the four ABFs, but this result was difficult to accurately interpret due to previously reported autoactivation ((Kumimoto et al.,

2010), compare to empty vector interaction in Figure 3.3A). Previous reports indicate that NF-YC subunits also mediate bZIP interactions in fungal and mammalian systems (Yoshida et al., 2001; Singh et al., 2011), suggesting broad evolutionary conservation of these interactions in the eukaryotic lineage.

Although the NF-YB2 by ABF3 interaction was reproducible in the directed Y2H tests (Figure 3.3B), no other NF-YB by bZIP interaction was detected. This result was unanticipated because NF-YB2 and NF-YB3 share nearly 70% amino acid identity over their entire proteins. Further, their central conserved domains, the 95AA histone fold motif (HFM), are 93% identical and 100% similar (Supplemental Figure 3.1). This led to the hypothesis that regions outside of the conserved domain in NF-YB2 are responsible for its unique ability to interact with ABF3.

To test this hypothesis, we dissected the NF-YB2 protein into three different regions: the N-terminal region up to the conserved domain (Figure 3.4A, B2N, AA 1-25), the conserved HFM (B2HFM, AA 26-121) and the C-terminal region (B2C, AA 122-190). Constructs containing each of the regions were tested in Y2H for interaction with ABF3. None of the partial NF-YB2 clones showed interaction with ABF3 (Figure 3.4A), although the conserved histone fold motif of NF-YB2 still retained the ability to interact with NF-YC proteins (data not shown). This led to the hypothesis that one of the ends of NF-YB2, in conjunction with the HFM, would drive the interaction with ABF3. Clones were

generated from the start of NF-YB2 through the HFM (B2N + B2HFM, AA 1-121) and starting from the first AA of the HFM through the end of the protein (B2HFM + B2C, AA 26-190). Like the partial constructs above, neither of these constructs interacted with ABF3 (Figure 3.4A). Thus, if the N or C terminal ends were required for NF-YB2 specificity for ABF3 interactions, these appeared to still require the context of the full length protein.

To explore this latter idea, we created chimeric proteins between NF-YB2 and NF-YB10. We chose NF-YB10 because it has highly divergent terminal ends outside of the HFM and also showed no interaction with ABF3 (Figure 3.4B and Supplemental Figure 3.1). The constructs contained either the N-terminal region of NF-YB2 or NF-YB10 through the first half of the HFM fused to the C-terminal region of the other NF-YB (Figure 3.4B). Interestingly, the NF-YB2 N-terminal/NF-YB10 C-terminal chimeric protein was able to interact with ABF3, although more weakly than the native full length NF-YB2. This is in contrast to the complementary construct (NF-YB10 N-terminal/NF-YB2 C-terminal), which did not interact (Figure 3.4B). Collectively, these data suggest that the N-terminal domain of NF-YB2 is necessary but not sufficient for the specific interaction with ABF3. A previous report in Arabidopsis also suggested that bZIP28 interactions with NF-Ys were subunit specific (NF-YB3) and additionally required the initial formation of an NF-YB3/NF-YC2 dimer (Liu and Howell, 2010). Thus, additional interactions may be masked in our two way directed tests.

NF-YC have opposing roles in ABA responses

Our bioinformatic analyses and Y2H screens suggest that NF-Y/bZIP interactions may be commonplace. These findings led us to hypothesize that, like many bZIPs, NF-Y are also involved in ABA responses. Warpeha et al. (Warpeha et al., 2007) previously demonstrated that several Arabidopsis *nf-y* mutants have delayed germination in the presence of ABA. We extended these analyses by first testing the germination of *nf-y triple* mutants on non-ABA containing media, where no significant differences to control were observed (Figure 3.5A). Conversely, on media supplemented with ABA, germination inhibition was significantly reduced in *nf-yc triple* mutants compared to Col-0 control plants (Figure 3.5B-C,J). This result was similar to previous observations of both *abf1* or *hy5* mutants (Sharma et al., 2010; Chen and Xiong, 2011).

Although *NF-YC3*, *NF-YC4* and *NF-YC9* have overlapping functionality in photoperiod-dependent flowering, it was unclear whether the same would be true for these ABA related phenotypes. In fact, it was previously shown that *nf-yc4* single mutants were hypersensitive to ABA (Warpeha et al., 2007) - the opposite of what we observed for the *nf-yc triple* mutant which includes the same *nf-yc4-1* loss of function allele. Therefore, we tested the germination of all possible single and double mutant combinations of *nf-yc3*, *nf-yc4*, and *nf-yc9* (see Materials and Methods for allele designations).

The response of *nf-yc3 nf-yc9* double mutants on ABA media was non-distinguishable from the *nf-yc triple*, indicating that *NF-YC4* does not normally play a role in repressing ABA-mediated germination (Figure 3.5D-F,J). As was previously reported (Warpeha et al., 2007), *nf-yc4* single mutants were hypersensitive to ABA in our germination assays (Figure 3.4H,J). Like *nf-yc4*, the *nf-yc3 nf-yc4* and *nf-yc4 nf-yc9* double mutants were also slightly hypersensitive to ABA and germinated later than controls (Figure 3.5E,J). The opposite is true in *nf-yc triple* mutants where the early germination phenotype of the *nf-yc3 nf-yc9* double mutant is observed (Figure 3.5B). To extend these ABA analyses beyond germination, we additionally measured cotyledon greening and root elongation.

For cotyledon greening assays, seeds were sown on media containing 1μM ABA and the percentage of plants with open green cotyledons was scored after 10 days of growth in continuous light (Kim et al., 2004). As predicted from the germination data, *nf-yc3 nf-yc9* double and *nf-yc triple* mutants performed much better than controls - after 10 days nearly 100% of these plants had open green cotyledons compared to less than 20% for parental Col-0 (Figure 3.5K-L). Poor greening for *nf-yc4* single and *nf-yc3 nf-yc4* double mutants also correlated with the germination phenotypes. However, *nf-yc9* single mutants, which showed no significant differences in germination, had a significantly greater number of seedlings that greened (Figure 3.5K). Additionally the *nf-yc4 nf-yc9* double

mutants greened similarly to *nf-yc9* single mutants. This is in contrast to the ABA germination phenotype exhibited by the *nf-yc4 nf-yc9* double mutant where the *nf-yc4* phenotype was observed. In contrast to the cotyledon greening, *NF-YC* mutants showed no clear root elongation differences on media supplemented with ABA (Supplemental Figure 3.2). These observations indicate that *NF-YC* not only have opposing roles in ABA responses, but also have separable roles in germination and post germination growth in the above ground plant.

NF-YB2 and *NF-YB3* overexpression significantly delays germination

Because of the known physical interactions between NF-YB2/NF-YB3 and NF-YC3/NF-YC4/NF-YC9 (Kumimoto et al., 2010), as well as our demonstration that NF-YB2 can physically interact with ABF3, we additionally tested these NF-YBs in ABA germination assays and root elongation assays. As with *nf-yc3*, *nf-yc4*, and *nf-yc9* mutants, we measured no root growth defects in response to ABA for the *nf-yb2* and *nf-yb3* mutants (Supplemental Figure 3.2). Further, we found that neither *nf-yb2* or *nf-yb3* single knockdown mutants, nor *nf-yb2 nf-yb3* double mutants showed any significant alteration in germination on either regular media or ABA-supplemented media (Figure 3.6A-B; note that the same *nf-yb* alleles have clear late flowering phenotypes (Cai et al., 2007; Kumimoto et al., 2008; Cao et al., 2011).

We additionally used the 35S promoter from the cauliflower mosaic virus (Kay et al., 1987) to overexpress both *NF-YB* genes in transgenic plants [Note that the same experiment with the *NF-YC* genes was not possible due to the consistent inability to obtain stable overexpressing transgenic lines]. Stable, single insertion, third generation *NF-YB* overexpressing plant lines were chosen. When assayed on non-ABA growth media, *p35S::NF-YB2* and *p35S::NF-YB3* seeds reached 100% germination approximately 24 hours later than control plants (Figure 3.6C). In the presence of 1μM ABA, *p35S::NF-YB2* and *p35S::NF-YB3* seeds were much later germinating and never reached 100% germination (typically peaking at ~50% total germination by 120 HAI, Figure 3.6D). These data demonstrate that *NF-YB2* and *NF-YB3* overexpression can significantly alter seed germination responses, but knockdown data suggests this may not be their normal biological role. These results are similar to what is observed with *abf* mutants, where single loss of function alleles did not show strong phenotypes, but *ABF* overexpression could inhibit germination on ABA media (Kang et al., 2002; Kim et al., 2004; Yoshida et al., 2009). Thus, higher order mutants combining *nf-yb2*, *nf-yb3*, and additional *nf-yb* knockdown alleles might uncover a biological role in seed germination for *NF-YB2* and *NF-YB3*.

To help sort through these possibilities, we examined the tissue-specific expression of *NF-YB2* and *NF-YB3* in imbibed seeds using previously described transgenic plant lines expressing the reporter gene β-glucuronidase

(GUS) driven by native *NF-Y* promoters (*pNF-Y::GUS* lines, (Siefers et al., 2009)). If these *NF-Y* were likely to be normally involved in seed germination, expression would be expected in either the developing embryos or endosperm. As controls, we also examined the *pNF-Y::GUS* lines for *NF-YC3*, *NF-YC4*, and *NF-YC9* where our loss of function data already clearly demonstrated a biological role in germination. As expected from the *nf-yc* mutant data above, all three *NF-YC* genes were consistently expressed in seeds, including both the embryos and endosperm layer (Figure 3.7C-F, I-L). Although there were variations in specific expression patterns, all three *NF-YC* had expression throughout the embryonic root with the strongest GUS staining in the meristematic regions. Additionally, staining was typically stronger in the vascular regions of the cotyledons than the remainder of the seed leaf (this contrast was strongest for *NF-YC3*, Figure 3.7J). However, most *pNF-YB2::GUS* seeds showed no staining in the embryo or endosperm (Figure 3.7B, H) and *pNF-YB3::GUS* seeds showed only weak and inconsistent staining in the embryo and endosperm (Figure 3.7C, I). The observed expression patterns for the promoter fusion constructs are consistent with publically available seed microarray data (Supplemental Figure 3.3 using eFP Browser (Winter et al., 2007; Bassel et al., 2008)). The addition of ABA did not significantly change the expression patterns for any of the constructs or mRNA patterns from public microarray studies (data not shown). These data support the hypothesis that NF-YB2 and NF-YB3 are not normally the major NF-YB components of the

germination-influencing NF-Y complexes. Nevertheless, both can clearly and reproducibly alter germination when ectopically expressed in seeds.

ABA and germination marker genes have altered expression in *NF-Y* mutants

To further test *NF-Y* roles in ABA signaling and germination, we used qPCR to examine transcript levels of several genes that are well-known to be responsive to bZIPs - *ABI3*, *ABI5*, *AIA*, *AIL*, *RAB18*, and *RD29B* (Chen et al., 2008; Yoshida et al., 2009; Umezawa et al., 2010). For these assays we examined transcripts from imbibed seeds after 24h of light exposure (see Materials and Methods). Based on our phenotypic data, we chose to compare the *nf-yc3 nf-yc9* double mutants to the *nf-yc4* single mutant. Additionally, we examined gene expression in the ABA-hypersensitive *p35S::NF-YB2* and *p35S::NF-YB3* lines. Consistent with ABA-insensitivity phenotypes, the germination inhibitors *ABI3* and *ABI5* were downregulated ~5-10 fold in *nf-yc3 nf-yc9* mutants (Figure 3.8A-B). The ABA-hypersensitive lines *nf-yc4*, *p35S::NF-YB2*, and *p35S::NF-YB3* all had less dramatic, but increased levels of *ABI3* expression. *ABI5* expression only increased in the *NF-YB* overexpressing lines. Collectively, these data are also consistent with previous research showing that *ABI3* expression is positively regulated by *LEC1* (*NF-YB9*, (Kagaya et al., 2005; To et al., 2006)). For the ABA-regulated genes *AIA*, *AIL*, *RAB18*, and *RD29B*, we consistently measured downregulated expression in the *nf-yc3 nf-yc9* mutants, ranging from -2.5 fold (*AIA*) to -14.3 fold (*AIL*, Figure 3.8C-F). Once again, the two *p35S::NF-*

YB lines consistently had the opposite response. For several genes the differences were quite dramatic. For example, in the *p35S::NF-YB2* transgenic seeds, *AIL* was upregulated 22.5 fold (Figure 3.8D) and *RAB18* was up 15.5 fold (Figure 3.8E). None of these ABA-regulated genes were strongly misregulated in *nf-yc4* mutants, although the general trend was unexpectedly slightly down for each gene other than *ABI3*. The lack of strong differences in *nf-yc4* may be due to the relatively weak ABA sensitivity phenotypes reported here and elsewhere (Warpeha et al., 2007). Future experiments examining higher order mutants, especially a double mutant between *NF-YC4* and its apparent paralog *NF-YC1* (Siefers et al., 2009) may improve the resolution of this analysis.

Discussion

Through bioinformatics and mutant analyses we add to the growing body of evidence that NF-Y and bZIP transcription factors cooperatively regulate similar subsets of genes and, thereby, some of the same plant processes. It also appears likely that NF-Y and bZIP form higher order regulatory complexes capable of integrating inputs from many signaling pathways. Examination of the *cis*-regulatory regions of mis-expressed genes in *nf-yc triple* mutants uncovered an over-representation of G-box and ABRE-like bZIP binding motifs. ABRE and G-box elements are common in the promoters of genes responsive to abiotic stress and light (Yamaguchi-Shinozaki and Shinozaki, 2005; Kim, 2006). Studies of ABRE containing promoters demonstrated that single ABRE

sequences are not sufficient to induce transcriptional activation and a coupling element is required for induction of ABA responsive genes (Shen et al., 1996). Our data suggests that the NF-Y-bound *CCAAT* box might play the role of the ABRE coupling element for some bZIP-responsive promoters.

In Arabidopsis, Liu and Howell show that a G-box containing promoter (ERSE-I) required intact *CCAAT* and *CACGTG* (G-box) elements for full activation in response to ER stress (Liu and Howell, 2010). Further support that the *CCAAT* box may act as a coupling element to the G-box comes from outside the plant kingdom. Motifs similar to the ABRE were found to be over-represented in *NF-Y* bound mammalian promoters (Dolfini et al., 2009). Further, in response to ER stress, mammalian NF-Y must bind to the *CCAAT* box in ERSE containing promoters before the bZIP protein ATF6 can bind an adjacent G-box like element (Yoshida et al., 2001).

In contrast, Yamamoto *et al.* demonstrated that NF-YB, NF-YC, and bZIP proteins collectively activate the ABRE-containing promoter of *CRUCIFERIN C* (*CRC*), but claim that this activity does not require the *CCAAT* box sequence (Yamamoto et al., 2009a). Further, the addition of NF-YA subunits to their protoplast assays inhibits *CRC* expression. These data suggest a non-canonical use of the NF-YB/NF-YC dimer where NF-YA subunits are not involved in the final transcriptional complex. In rice, OsMADS18 was shown to interact with an NF-YB/NF-YC dimer, without the NF-YA, suggesting that some

NF-Y may have evolved to form atypical complexes in plants (Masiero et al., 2002). Atypical NF-Y complexes (i.e., lacking NF-YA) would be unlikely to bind *CCAAT* boxes because the NF-YA subunit is thought to make all the direct physical contacts with the *CCAAT* nucleotide sequence (Xing et al., 1993; Romier et al., 2003). Nevertheless, while it is possible that some NF-Y might not bind the actual *CCAAT* box, they were still necessary for bZIP67 activation of CRC expression.

NF-Y and bZIP proteins not only bind the same promoters, but can also physically interact (Yamamoto et al., 2009a; Liu and Howell, 2010). Here we show that NF-YC subunits interact with different bZIP proteins fairly indiscriminately in directed Y2H assays. This result is consistent with data from animals and fungi where the NF-YCs appear to make the primary contacts with bZIP proteins (Yoshida et al., 2001; Singh et al., 2011). While NF-YC proteins indiscriminately bound bZIP proteins in our Y2H assays, the NF-YB proteins were more selective. This suggests that NF-YBs may play a role in discriminating which bZIP binds to an NF-YB/C dimer or the full complex. In addition, we demonstrate that regions outside of the highly conserved HFM are likely driving the preference of NF-YB for bZIP partners. This observation is of interest because it is consistent with protoplast assays where only the closely related NF-YB6 and NF-YB9 could interact with bZIP67 to activate the CRC promoter, as well as the finding that bZIP28 only interacts in complexes containing NF-YB3 (Yamamoto et al., 2009a; Liu and Howell, 2010). Together,

these data raise an intriguing hypothesis for the large expansion and maintenance of NF-YB subunits in plants as compared to animal and fungal systems. It is possible that NF-YB have evolved specific interactions with bZIP proteins whereas in other systems the interactions are more general.

In addition to promoter analyses and Y2H assays, we show that NF-Y mutant plants have morphological phenotypes related to ABA signaling and that several well-known marker genes are misregulated in the various mutant backgrounds. Initial germination studies with *nf-yc triple* mutants showed that they are less sensitive to ABA treatments. This was surprising because the *nf-yc4* single mutant, one of the mutants comprising the *nf-yc triple*, was previously reported as being more sensitive to ABA in germination assays (Warpeha et al., 2007). We then tested all possible double mutant combinations as well as the single mutants of *NF-YC3*, *NF-YC4*, and *NF-YC9*. Out of this analysis an epistatic relationship between *NF-YC3*/*NF-YC9* and *NF-YC4* emerged. *nf-yc3/nf-yc9* double mutants showed identical ABA germination phenotypes to *nf-yc triple* mutant plants. This suggests that NF-YC3 and NF-YC9 are interchangeable as negative regulators of germination, and NF-YC4 does not have overlapping functionality in this process. The fact that NF-YC3 and NF-YC9 work as functional equivalents is not surprising as their amino acid sequences are 100% identical throughout the conserved DNA binding and NF-YA/NF-YB interaction domains (Kumimoto et al., 2010). NF-YC4 is more divergent in these domains and in agreement with previous reports (Warpeha et

al., 2007) we demonstrated that *nf-yc4* single mutants are late germinating on ABA-containing media. The epistasis analysis between the late germinating *nf-yc4* single mutant and early germinating *nf-yc3/nf-yc9* double mutant revealed that a functional NF-YC3 or NF-YC9 protein is required for NF-YC4 to act as a positive regulator of seed germination.

The epistasis of NF-YC3/NF-YC9 to NF-YC4 suggests a simple linear genetic pathway where NF-YC4 is upstream and possibly directly regulates NF-YC3 and NF-YC9. Another possibility is that NF-YC3, NF-YC4 and NF-YC9 are acting competitively at single hubs to regulate the ABA germination response. Similar functional antagonism between closely related family members has been seen with the seed specific bZIPs, ABI5 and EEL (Enhanced EM Level, bZIP12, (Bensmihen et al., 2002)). Mutant analysis revealed that EEL is a negative regulator of embryogenesis-abundant genes and is dependent on the presence of the positive regulator ABI5. Interestingly, instead of a simple linear pathway where EEL regulates ABI5, it was shown that ABI5 and EEL could interact and possibly compete for the same ABRE promoter elements in embryogenesis-abundant genes. It will be interesting to determine if positive and negative regulatory NF-Y complexes can compete for the same elements. In addition it stands to reason that bZIP proteins could also be involved in these complexes.

In an attempt to determine the composition of biologically active NF-Y complexes related to ABA signaling, we additionally tested *nf-yb2* and *nf-yb3* mutants for their roles in germination. Both of their encoded proteins interact *in vivo* with NF-YC3 and NF-YC4 to regulate flowering, but these assays only utilized seedlings (Kumimoto et al., 2010). *nf-yb2* and *nf-yb3* loss of function mutants showed no clear difference to controls in ABA germination assays, but gain of function *p35S::NF-YB2* and *p35S::NF-YB3* lines showed strong repression of germination in the presence of ABA. While these gain of function phenotypes suggest a role for NF-YB2 and NF-YB3, their lack of native expression in the endosperm/embryo and the absence of loss of function phenotypes suggest other NF-YB act natively in the germination complex. It is likely that NF-YCs partner with LEC1 and L1L (NF-YB6 and NF-YB9, respectively) as these NF-YB proteins are well-known, embryo-specific regulators. Similar to *nf-yc3 nf-yc9* double mutants, *lec1* mutant plants are less sensitive to ABA in germination assays (West et al., 1994). In addition, NFYC3, NF-YC4 and NF-YC9 can all interact with LEC1 and L1L in directed Y2H assays (Bourgarel et al., 1999; Buschlen et al., 2003) and we consistently isolate LEC1 and L1L in library screens using NF-YC9 as bait (BFH, CLS, RWK unpublished data). Although *nf-yc3 nf-yc9* and *lec1* mutants share reduced ABA sensitivity in germination assays, *nf-yc3 nf-yc9* mutants do not have the *lec1* leafy cotyledon phenotypes. This suggests that there may be additional, as yet unidentified NF-YC(s) involved in embryogenesis.

Although NF-Y can interact with bZIP proteins, and can regulate the expression of a similar subset of targets, it remains to be determined how and if these complexes form *in planta.* With the recent discovery of the long sought ABA receptor, it has become clear that Group A bZIP transcription factors are directly activated through phosphorylation in the presence of ABA (Cutler et al., 2010; Fujita et al., 2011). In contrast to these bZIP proteins, relatively little is known about NF-Y complexes. How NF-Y activity is regulated and what *cis*-regulatory elements they are capable of binding remains to be determined for the plant lineage.

In this study we begin to uncover the complex and sometimes antagonistic roles that NF-Y play in ABA signaling. In addition, we add to the mounting evidence that plant, animal, and fungal NF-Y interact with bZIPs to form multi-protein, transcription-regulating hubs to affect gene expression. Future studies describing the regulation and formation of these complexes will further our understanding of how plants integrate multiple signals to fine tune growth and development.

Materials and Methods

Plant Materials

All plant material used were of the Col-0 ecotype. All mutants are combinations of the following alleles; *nf-yc3-1* (SALK_034838), *nf-yc4-1* (SALK_032163), *nf-yc9-1* (SALK_058903), *nf-yb2-1* (SALK_025666), *nf-yb3-2* (SALK_150879). The

p35S:NF-YB2 and *p35S:NF-YB3* lines and were previously described (Kumimoto et al., 2008; Kumimoto et al., 2010; Cao et al., 2011).

All germination and root growth assays were performed using seeds that were collected from plants grown concurrently in the same growth chamber under standard long day conditions (16h day/8h night, 90μmol m^{-2} s^{-1}, 22°C). Plants for matched seeds were grown in media containing equal parts Farfard C2 Mix and Metromix 200 supplemented with 40 g Marathon pesticide and dilute Peter's fertilizer (NPK 20:20:20).

Germination and root growth assays

Matched seed sets were harvested and allowed to after ripen for between 2 to 4 months before use in assays. Seeds were surface sterilized and plated onto Gamborg's B5 media (SIGMA, St. Louis, MO, Cat#G5893) or B5 media containing the appropriate amount of (+/-) ABA (SIGMA, Cat#A4906). For germination and cotyledon greening assays, plates were cold stratified for 3 days and placed in continuous light at 90μmol m^{-2} s^{-1} at 22°C in a Conviron model ATC13 chamber. Seeds were scored every 12 hours post incubation for visible radical emergence as a proxy for seed germination. Greening was assayed by counting plants with open green cotyledons on day 10 (Kim et al., 2004). Plants for root growth assays were sown to B5 plates and incubated under long day conditions for four days before transfer to plates supplemented with ABA. Plates were oriented vertically for an additional seven days. All plates

were photographed and primary root length was measured with image J (Abramoff et al., 2004). All germination, greening, and root growth assays were repeated a minimum of three times with at least two independent sets of matched seeds with consistently similar results. All statistics were performed in either INSTAT or Prism (GraphPad Software - La Jolla, CA).

Promoter Analysis

Over representation analyses on nuclear encoded genes from the *nf-yc triple* were performed on genes misregulated ≤-1.5 and ≥1.5 fold ($p<.05$) compared to Col-0. Details of the microarray experiment and public access to MIAME compliant data were previously reported (Kumimoto et al., 2010). Because the purpose of the current analysis was hypothesis generation (which were later tested), we relaxed the stringency of the microarray analysis here by removing the Benjamini-Hochberg false positive correction. For MEME de-novo motif discovery, -500bp of upstream sequence of the misregulated genes was obtained using the bulk data retrieval tool at The Arabidopsis Information Resource (TAIR, www.arabidopsis.org). Upstream DNA sequences were fed to the MEME program and analyzed using the following parameters: motif width between 5 and 12bp, any number of repetitions of motifs, and search for up to 6 motifs. All other options were left as default (http://meme.nbcr.net/meme4_5_0/intro.html, (Bailey et al., 2006)). Motifs discovered through MEME analysis were then compared against known transcription factor binding sites from Jasper, Transfac and Uniprobe using the

TOMTOM motif comparison tool (Gupta et al., 2007). Positions of *CACGTG* motifs relative to the transcriptional start were adapted from the MEME analysis. The same misregulated gene list was input into the Athena analysis suite using a 500 max bp upstream cutoff and, otherwise, default settings (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl, (O'Connor et al., 2005)).

Protein-Protein Interaction Network

Individual protein-protein interaction networks were built for NF-YC3, NF-YC4 and NF-YC9 using GeneMANIA (http://www.genemania.org, (Mostafavi et al., 2008; Warde-Farley et al., 2010)). Selection criteria to develop the network map in GENEMANIA were predicted interactions and physical interactions with a 50 gene output. Default settings in GeneMANIA were used for network weighting. The individual network maps built in GeneMANIA for NF-YC3, NF-YC4 and NF-YC9 were downloaded as text files and combined to build a protein interactome in Cytoscape 2.8.0 (http://www.cytoscape.org, (Cline et al., 2007)). Data from Y2H library screens and directed Y2H assays done in the Holt lab and published interactions of NF-YC3, NF-YC4 and NF-YC9 (Kumimoto et al., 2010) were manually added to the protein interactome in Cytoscape.

RNA isolation and qPCR

Total RNA was isolated from 20 mg of matched, stratified seeds using the E.Z.N.A. Plant RNA Kit per the manufacturer's instructions for difficult samples

(Omega Biotek, Inc., Norcross, GA, CAT#R6827-01). Prior to RNA extraction, seeds were sown on beds of Whatman paper saturated with liquid Gamborg's B-5 media, cold stratified for 2 days, and exposed to 24 hours of continuous light at 22°C before harvesting. To completely remove genomic DNA, samples were DNAse treated on E.Z.N.A. RNA isolation columns (Omega Biotek, CAT# E1091). Quality and quantity of RNA samples were confirmed by spectrophotometry (Thermo Scientific, Waltham, Massachusetts, NanoDrop™ 1000). First-strand cDNA synthesis was performed using the Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, California, Cat#18080-051) with supplied oligo dT primers. qPCR was performed as previously described (Kumimoto et al., 2008), except we used an Applied Biosystems Prism 7500 analyzer (Life Technologies, Carlsbad, California) and the Fermentas Maxima SYBR Green qPCR Master Mix (Fermentas, Glen Burnie, Maryland, Cat#K0222). For each genotype, we analyzed three independent, biological replicates in two separate experiments with similar results. All samples were normalized to the constitutively expressed gene At2g32170 as previously described (Czechowski et al., 2005). Sample comparisons were performed by the $2^{(-\Delta\Delta C_T)}$ method (Livak and Schmittgen, 2001), and errors (standard deviation) were computed as previously described (Nordgard et al., 2006). qPCR primers for *ABI3*, *ABI5*, *AIA*, *AIL*, *RAB18*, and *RD29B* (AT3G24650, AT2G36270, AT1G64110, AT3G17520, AT5G66400 and AT5G52300, respectively) were designed using Primer3 in Genious Pro 5.1.4

(www.genious.com, (Rozen and Skaletsky, 2000)). Primer sequences available upon request.

DNA manipulations

All target DNA fragments were generated by PCR using Pfu Ultra II (Agilent Technologies, Santa Clara CA, Cat#600670-51) and cloned into the Gateway™ entry vector pENTR/D-TOPO (Invitrogen, Carlsbad, California, Cat#45-0218). The full length coding regions of *NF-YB2*, *NF-YB3*, NF-YB10 *NF-YC3*, *NF-YC4*, *NF-YC9*, *ABF1*, *ABF2*, *ABF3*, *ABF4*, and *HY5* (AT5G47640, AT4G14540, AT3G53340, AT1G54830, AT5G63470, AT1G08970, AT1G49720, AT1G45249, AT4G34000, AT3G19290, and AT5G11260, respectively) were generated from Col-0 cDNA populations by standard methods (cloning primers available upon request). Partial clones of NF-YB2 and NF-YB10, as well as chimeric constructs between NF-YB2 and NF-YB10, were generated by PCR using Pfu Ultra II and cloned into the Gateway™ entry vector pENTR/D-TOPO. In partial clones that do not contain a native start codon an ATG was added in front of the region of interest. All constructs were sequenced and found to be identical to the expected sequences found at The Arabidopsis Information Resource database (Swarbreck et al., 2008).

Yeast Two-Hybrid Analyses

Gateway™ entry clones containing the full length coding regions of *NF-YC3*, *NF-YC4*, *NF-YC9*, *ABF1*, *ABF2*, *ABF3*, *ABF4*, and *HY5* were recombined using

the LR Clonase II reaction kit (Invitrogen, cat#56485) into ProQuest™ Two-Hybrid System vectors pDEST22 and pDEST32 (Invitrogen, Cat#PQ10001-01). All interactions were tested per the manufacturer's instructions. X-Gal assays were performed on nitrocellulose membranes containing yeast colonies frozen in liquid nitrogen and incubated at 37°C in Z-buffer containing X-Gal (5-Bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside, Gold Biotechnology, St. Louis, MO, cat#X4281L). Y2H library screening with NF-YB2 and NF-YC9 was previously described (Kumimoto et al., 2010) using published libraries (Burkle et al., 2005).

Gus staining and microscopy

All *pNF-Y::GUS* fusions used in seed coat expression assays were previously described (Siefers et al., 2009). Seed coats were dissected and GUS staining was performed as previously described (Malamy and Benfey, 1997; Perry and Wang, 2003). Seeds coats and embryos were visualized using a Zeiss AxioImager Z1m with Apotome (Zeiss - Oberkochen, Germany), using the DIC/BF filter and recorded using onboard Axiocam MRm and MRm5 camera. Images are compressions of a 3D Z-stack into a 2D image using the deconvolution and extended focus feature in the Axiovision software (version 4.8.1).

Acknowledgments

The authors thank Bing Zhang and Taylor Fore for use of the Zeiss AxioImager microscope and technical assistance. We additionally thank Krishna Suthar and Ashley Robbins for technical support in the lab.

Figures and Tables

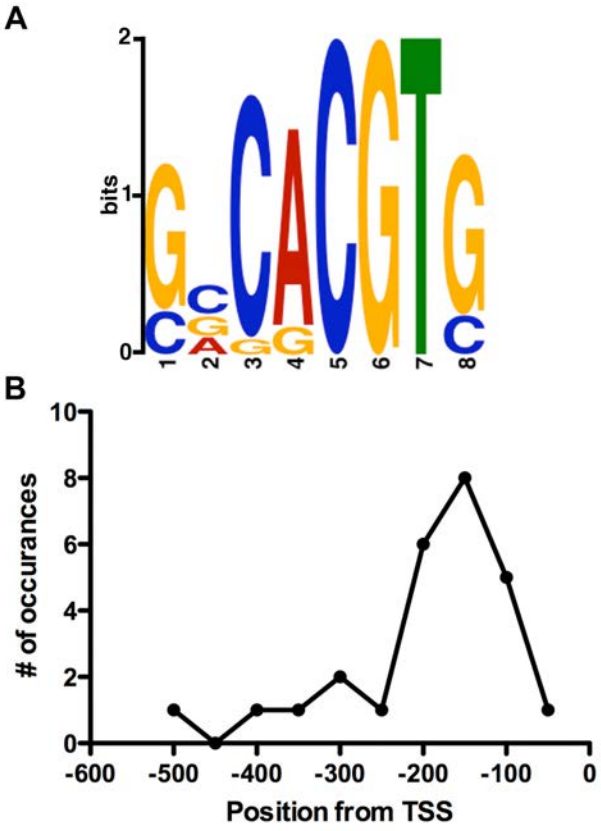

Figure 3.1. Misregulated genes in the *nf-yc triple* mutant have ABRE-like promoter elements. A) ABRE-like motif discovered through MEME analysis. B) Positional distribution of MEME motif within the promoter set. TSS - transcriptional start site. To help asses the relationships between Arabidopsis NF-Y proteins discussed here and below, note that phylogenetic trees were previously published (Gusmaroli et al., 2001, 2002; Siefers et al., 2009).

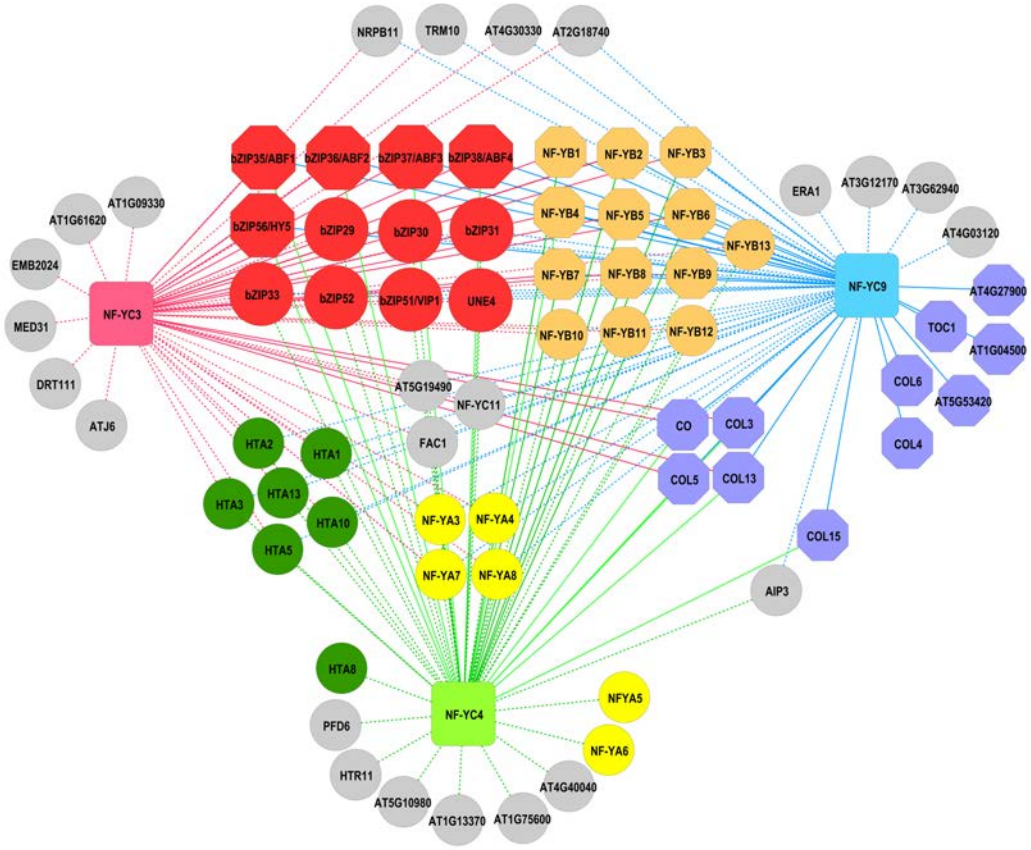

Figure 3.2. NF-YC3, NF-YC4 and NF-YC9 protein-protein interaction network. Both demonstrated and GeneMANIA predicted protein-protein interaction data for NF-YC3, NF-YC4, and NF-YC9 were visualized using Cytoscape (Cline et al., 2007). Predicted physical interactions are depicted as dashed lines, while demonstrated interactions ((Wenkel et al., 2006; Kumimoto et al., 2010) and this work) are depicted as solid lines. Input nodes NF-YC3, NF-YC4 and NF-YC9 are shown as squares. Circle nodes are those predicted data from GeneMANIA (Mostafavi et al., 2008; Warde-Farley et al., 2010). Octagonal nodes represent demonstrated physical interactions (e.g., Y2H, some shown in this report - see below). Related protein nodes are color coded as follows: red-bZIP; blue – CCT; green - H2A; orange/tan - NF-YB; yellow – NF-YA; Grey-unclassified interacting proteins.

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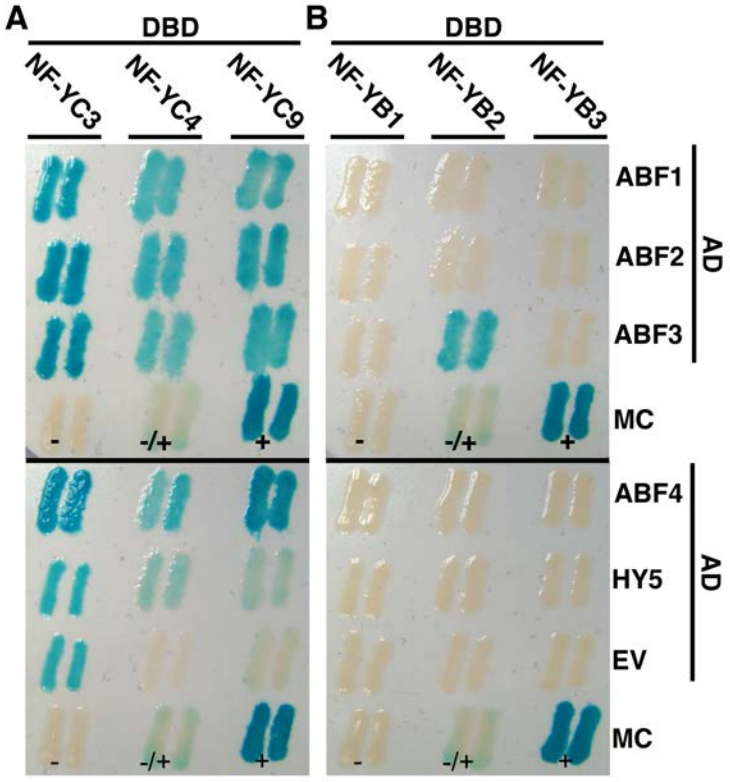


Figure 3.3. NF-YB and NF-YC subunits interact with bZIP transcription factors. Directed Y2H interactions between NF-YB or NF-YC subunits fused to DNA binding domains (DBD) and select bZIP proteins fused to activation domains (AD). Two independent colonies are shown for the activation of a β-galactosidase reporter gene (similar activation seen for two other reporters). A) NF-YC3, NF-YC4 and NF-YC9 interactions with ABF1-4 and HY5. B) NF-YB1, NF-YB2 and NF-YB3 interactions with ABF1-4 and HY5. MC = manufacturer's controls (+ = strong positive, +/- = intermediate positive, - = negative), EV = empty vector.

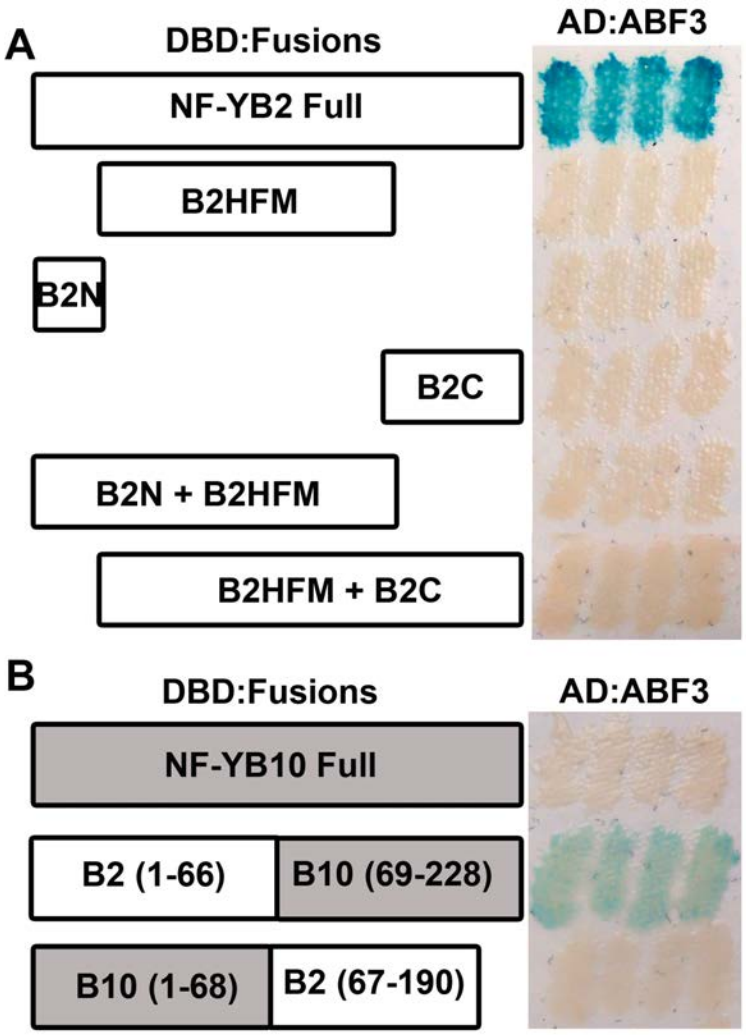


Figure 3.4. The N-terminal region of NF-YB2 is required for the ABF3 interaction. Y2H assays were performed between AD:ABF3 and DBD fused to: A) Full length NF-YB2 (AA 1-190), B2HFM (AA 26-121), B2N (AA 1-25), B2C (AA 122-190), B2N + B2HFM (AA 1-122), and B2HFM + B2C (AA 122-190); B) Chimeric constructs - full length NF-YB10 (AA1-220), NF-YB2/NF-YB10, and NF-YB10/NF-YB2.

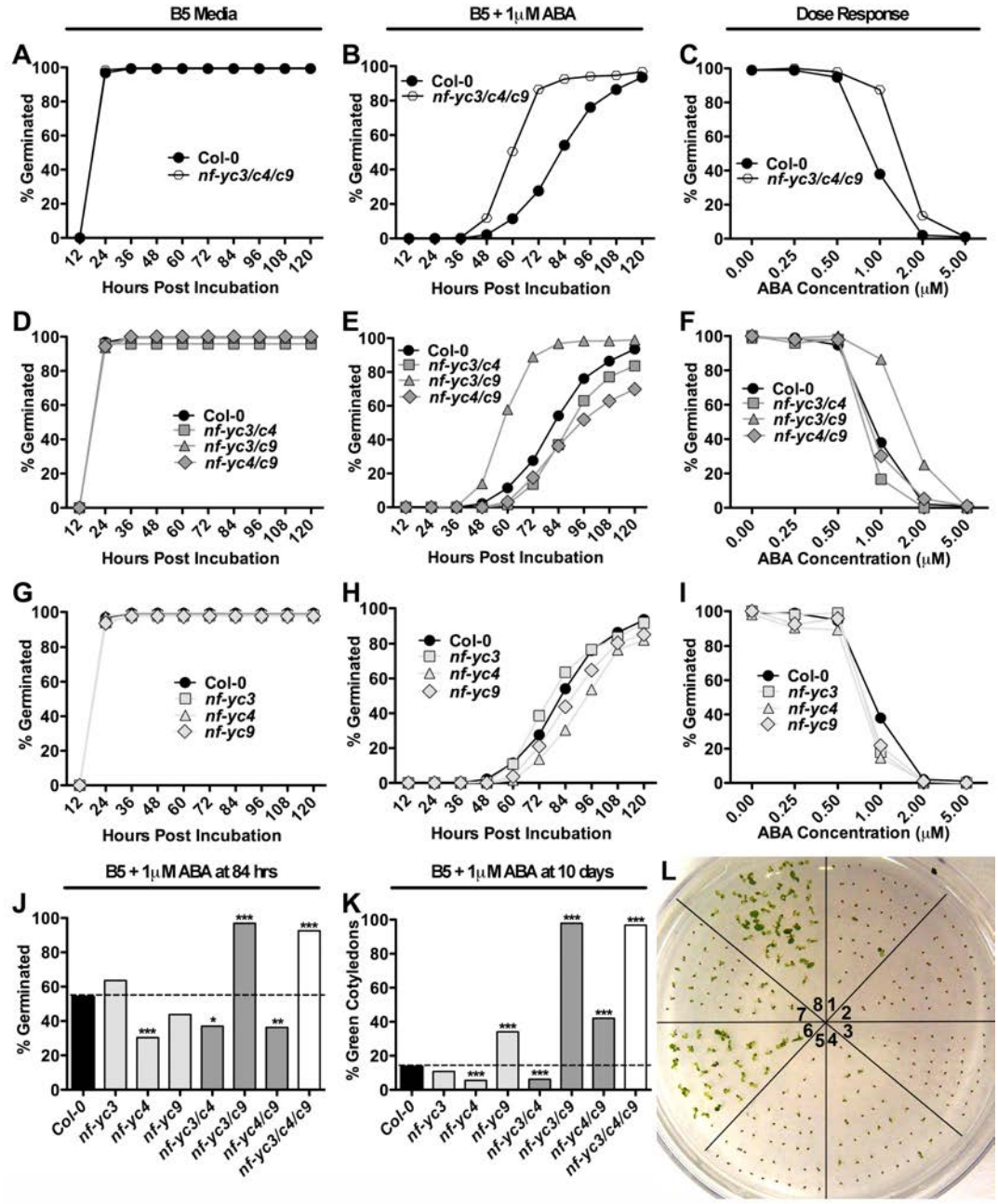


Figure 3.5. *NF-YC* mutants show opposing phenotypes in response to ABA. A-C) Germination of *nf-yc triple* mutants on B5 media, B5 media with 1μM ABA, and in response to variable ABA dosage at 84 hours, respectively. D-F) As in A-C for all *nf-yc* double mutant combinations and G-I) single mutants. J) Percent germination for all *nf-yc* mutant combinations on 1μM ABA at 84 hours. K) Percentage of plants with open green cotyledons at 10 days for all combinations of *nf-yc* mutants. L) Picture of 10 day old seedlings grown on 1μM ABA; 1 = Col-0, 2 = *nf-yc3*, 3 = *nf-yc4* , 4 = *nf-yc9*, 5 = *nf-yc3/c4,* 6 = *nf-yc3/c9,* 7 = *nf-yc4/c9,* 8 = *nf-yc triple*. Asterisks for J-K represent Fisher's Exact Test p-values; * p<0.01, ** p<0.001, *** p<0.0001. Germination data is a compilation of two experiments (n=6 replicates per genotype) using independent sets of matched seeds. Each replicate contained at least 30 seeds.

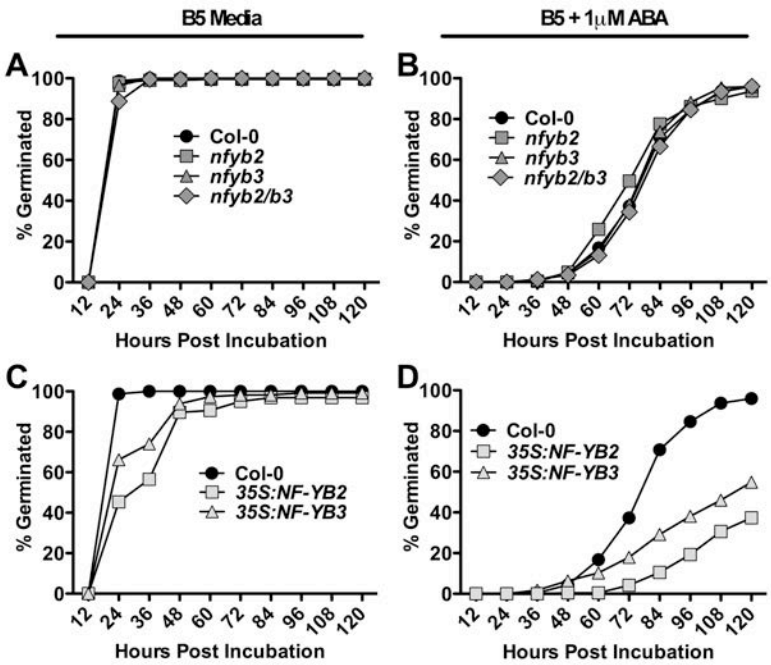


Figure 3.6. *NF-YB* overexpression results in late germination. A-B) *nf-yb2*, *nf-yb3* and *nf-yb2/b3* double mutants on B5 and B5+1μM ABA media. C-D) *p35S::NF-YB2* and *p35S::NF-YB3* on B5 and B5+1uM ABA media. Germination data is compilation of two experiments (total of n=6 replicates per genotype) using independent sets of matched seeds. Each replicate contained at least 30 seeds.

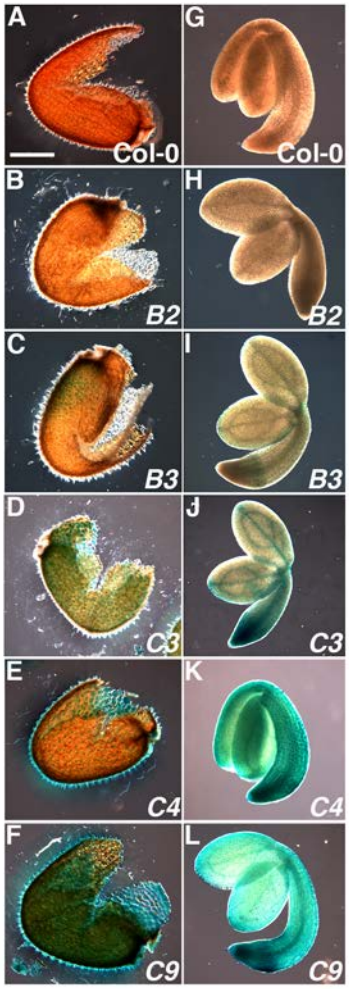


Figure 3.7. *NF-YC* are strongly expressed in embryos and the endosperm 24 hours post incubation in light. A,G) Col-0, B,H) NF-YB2, C,I) NF-YB3, D,J) NF-YC3 E,K) NF-YC4 F,L) NF-YC9. Scale bar in (A) equals 200μm.

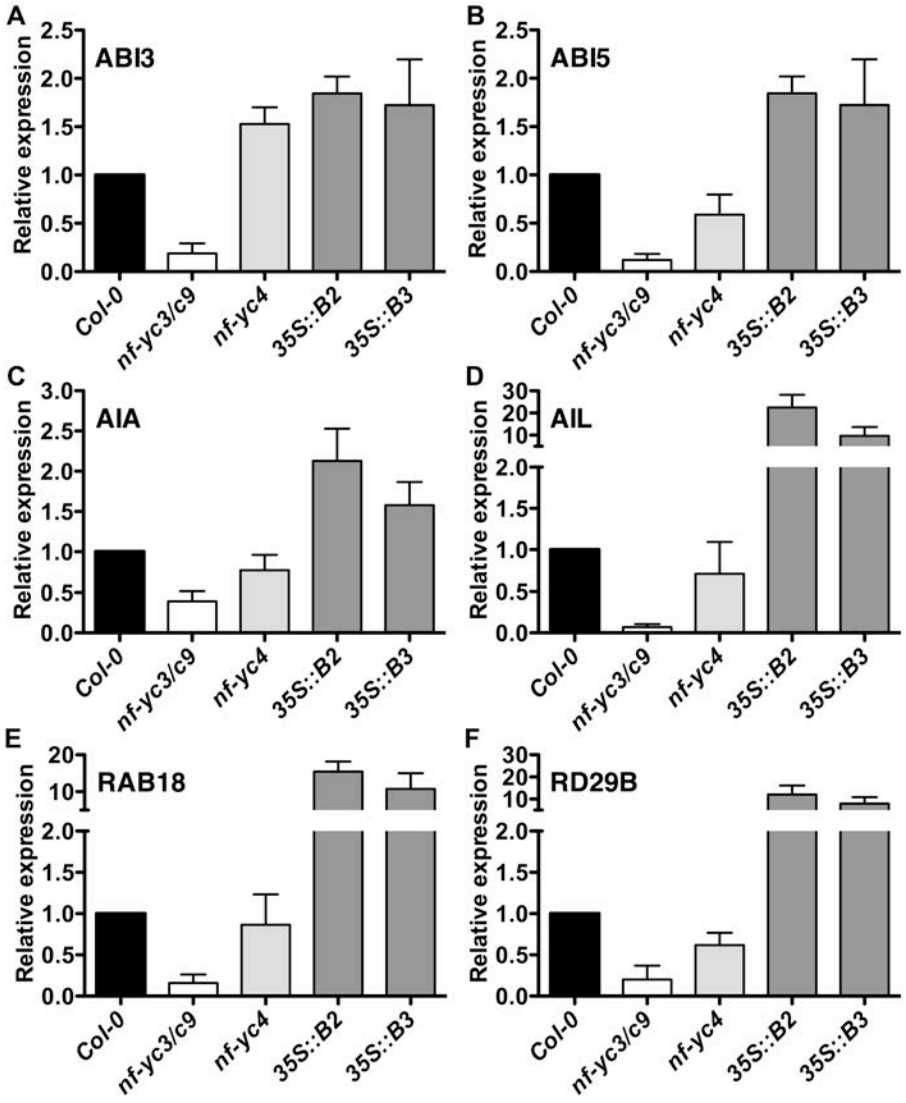
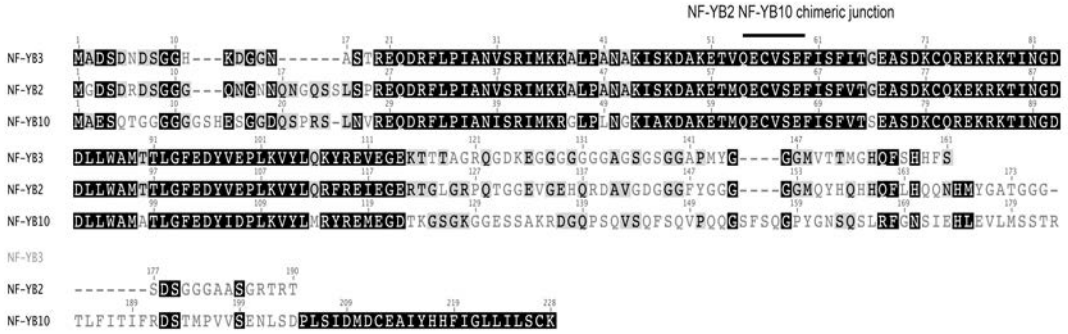
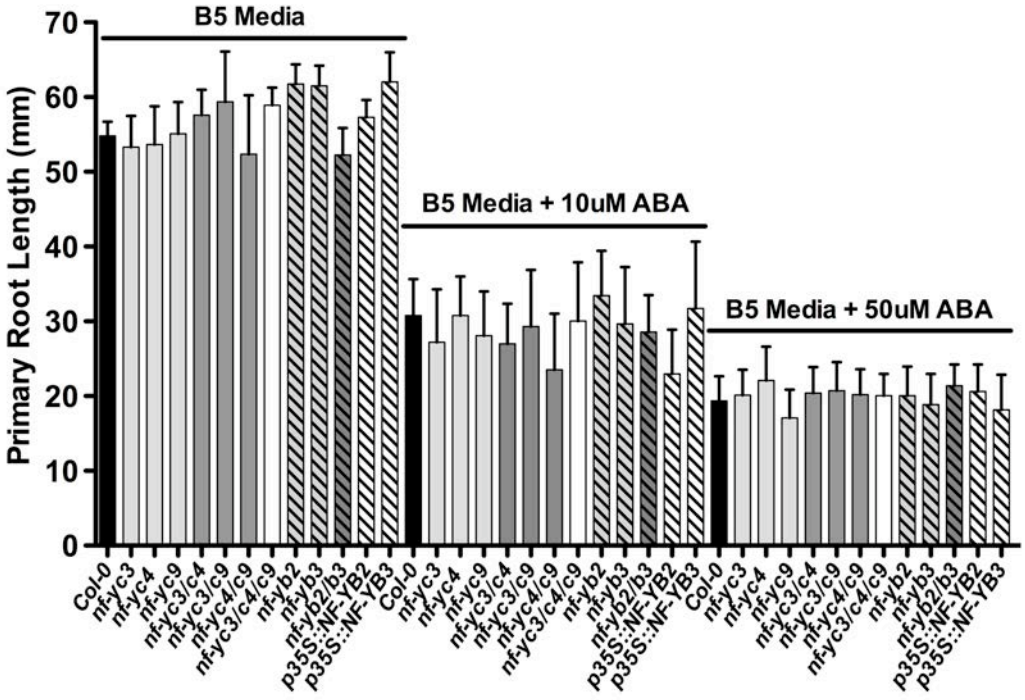


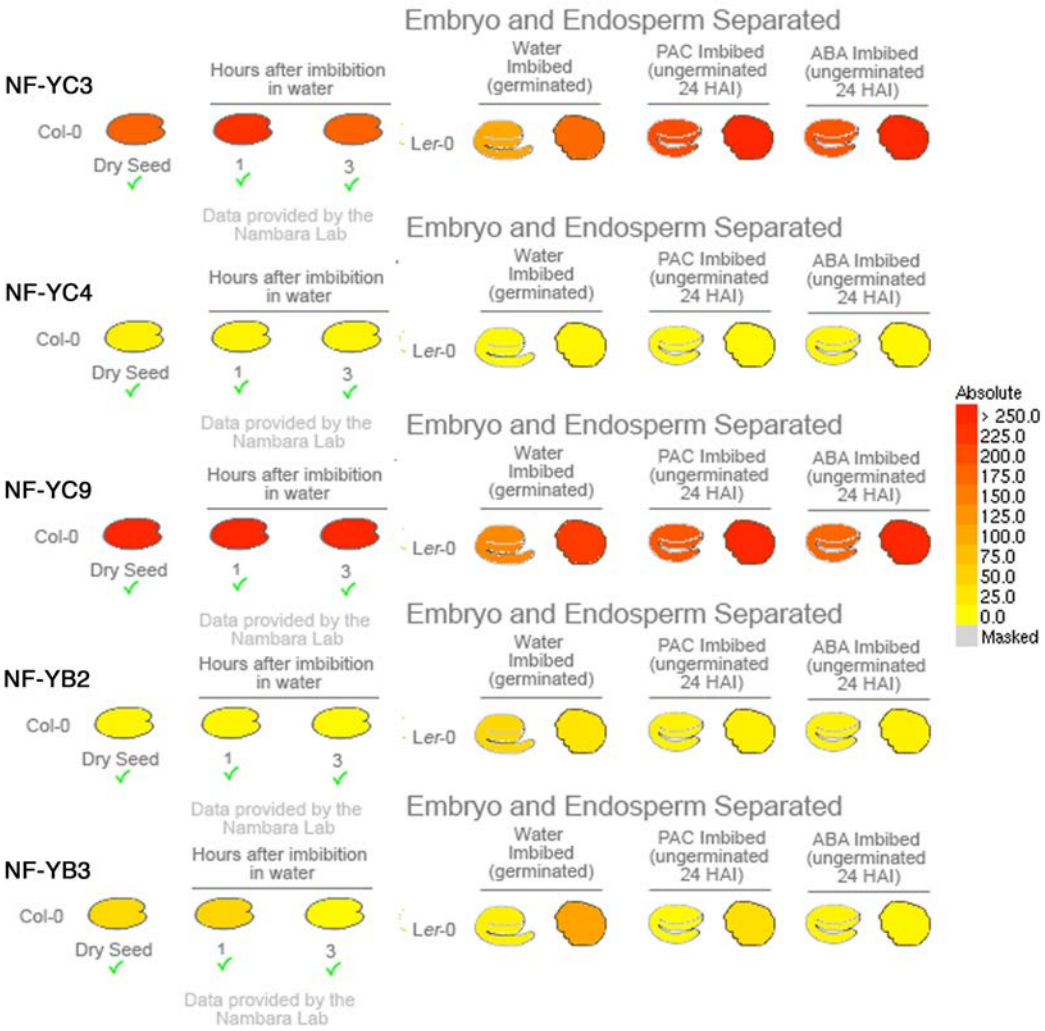
Figure 3.8. ABA related genes are misregulated in *NF-Y* mutant lines. Gene expression in 24hr post light incubation seeds analyzed by quantitative RT-PCR for A) *ABI3*, B) *ABI5*, C) *AIA*, D) *AIL*, E) *RAB18*, and F) *RD29B*. For each gene, the expression level in Col-0 was defined as 1. Data represent means and standard deviation of three replicates.



Supplemental Figure 3.1. Protein alignment of NF-YB2, NF-YB3 and NF-YB10. Full length amino acid sequences for NF-YB2, NF-YB3 and NF-YB10 were aligned and visualized using ClustalW within the software package Geneious Pro5.6 (www.geneious.com). The junction site used to create chimeric constructs between NF-YB2 and NF-YB10 is annotated.



Supplemental Figure 3.2. *NF-YB* and *NF-YC* mutants show no significant differences in root growth on ABA. Mutant lines were germinated and grown on B5 media for four days and then transferred to B5 media, B5 media + 10μM ABA, or B5 media + 50μM ABA and grown vertically for 7 days. Bars represent the mean primary root length (n ≥12 plants from 2 separate experiments). Error bars are 95% confidence intervals. No statistical significance between any samples on the same growth media was measured using ANOVA ($p>0.05$).



Supplemental Figure 3.3. Public microarray data visualized by eFP browser show *NF-YC3* and *NF-YC9*, but not *NF-YB2* and *NF-YB3* are expressed in seeds and during early germination. Absolute levels for *NF-YC3*, *NF-YC4*, *NF-YC9*, *NF-YB2*, and *NF-YB3* were queried in the eFP browser with a signal threshold of 250 (Winter et al., 2007; Bassel et al., 2008). Note that the lack of *NF-YC4* signal on public microarrays is likely due to problems with the probe (which is predicted to detect more than one gene) and not our GUS fusion. For example, according to public microarrays, *NF-YC4* is not expressed in leaf tissues, although we have previously published mRNA and protein data (using a native antibody) showing this is incorrect and there is a clear genetic requirement for *NF-YC4* in the leaf-initiated process of photoperiod-dependent flowering (Kumimoto et al., 2010).

Table 3.1. Over-represented *cis* motifs in promoters of genes misregulated in *nf-y triple* mutants.

Motif Name	Sequence	Database	% in set (#)	% in genome (#)	Pvalue
CACGTG	CACGTG	PLACE[a] (S000042)	30% (25)	9% (2797)	< 10e-7
ABRE like	BACGTGKM	AtcisDB[b]	28% (24)	12% (3861)	< 10e-4
ACGT ABRE	ACGTGKC	PLACE (S000394)	24% (20)	9% (2788)	< 10e-4
UPR Motif II	CCNNNNNNNNNNNNCCACG	PLACE (S000426)	8% (7)	2% (613)	0.001

[a] Database of Plant Cis-acting Regulatory DNA Elements, http://www.dna.affrc.go.jp/PLACE/ (Higo et al., 1999)
[b] Arabidopsis cis-regulatory element database, http://arabidopsis.med.ohio-state.edu/AtcisDB/ (Davuluri et al., 2003; Yilmaz et al., 2011)

Chapter 4: Nuclear Factor Y transcription factors regulate light signaling in *Arabidopsis thaliana*

Introduction

Environmental conditions have profound effects on the physiology and development of plants. Paramount among these conditions is the local light environment. In addition to being the primary energy source for photosynthesis, light plays a role in controlling developmental decisions such as seed germination, timing the floral transition, and dictating general plant architecture to maximize light capture. In the developing seedling, growth in response to light is termed photomorphogenesis and is described by inhibition of hypocotyl growth concurrent with the development of leaves and chlorophyll biosynthesis. Plants have evolved the ability to sense the quantity, quality (spectral composition), and duration of light received. To integrate these light signals for maximum fitness, plants must reprogram gene expression to achieve appropriate developmental or physiological outcomes.

There are four known photoreceptor protein classes in plants that measure the local light environment: phytochromes, cryptochromes, Light, Oxygen, Voltage (LOV) domain-containing, and *UV RESISTANCE LOCUS 8* **(**UVR8). Phytochromes absorb red and far red light, while cryptochromes absorb in the blue and UV-A spectrum. Phytochrome and cryptochrome have been studied for decades and mutant plants show phenotypes affecting photomorphogenesis, flowering time, shade avoidance response, and germination (Kami et al., 2010). LOV domain containing proteins, including PHOT1/2 and Zeitlupe, have been shown to be further blue receptors with roles

in chloroplast movement, stomatal aperture, and the circadian clock (Demarsy and Fankhauser, 2009). *UVR8* was recently shown to detect the UV-B part of the spectrum and is required for resistance to damaging ultra violet radiation (Heijde and Ulm, 2012). Through the activity of these light-absorbing proteins, signaling cascades are generated to affect transcription in plants, enabling proper developmental reprogramming.

In the model plant species *Arabidopsis thaliana* and *Oryza sativa*, approximately 20% of the genome shows differential expression between light and dark grown seedlings (Jiao et al., 2005). Transcriptions factors are found to be among the most over represented classes of early light regulated genes. Forward and reverse genetic screens have identified several transcription factors that are required for proper light responses (Tepperman et al., 2001; Jiao et al., 2005). Among the first discovered was *ELONGATED HYPOCOTYL 5* (*HY5*), a bZIP transcription factor. *HY5* mutants show partial etiolation (impaired photomorphogenesis) under all light conditions tested, indicating that *HY5* is a positive regulator of photomorphogenesis (Oyama et al., 1997; Ang et al., 1998b). HY5 acts down-stream of each class of photoreceptor making it a central hub for light signaling. *HY5* binds the G-box promoter element (Ang et al., 1998b; Chattopadhyay et al., 1998), which is found in many genes that are transcriptionally responsive to light. Recent genome levels studies of *HY5* implicate it in the regulation of approximately 1100 genes (Lee et al., 2007a).

Several B-Box (BBX) containing transcription factors also play a critical role in regulating light developmental processes. Interestingly, these highly related proteins have opposing roles in regulating light dependent growth. For example, *BBX18/DBB1a*, *BBX19/DBB1b*, *BBX24/STO* and *BBX32* have been shown to be negative regulators of photomorphogenesis (Indorf et al., 2007; Kumagai et al., 2008; Holtan et al., 2011), whereas *BBX21/STH2* and *BBX22/STH3*, like HY5, are positive regulators of photomorphogenesis (Datta et al., 2007; Datta et al., 2008). Both *BBX21* and *BBX22* have been shown to directly interact with HY5, suggesting that these B-Box proteins can modulate *HY5* activity.

A more distant B-box containing protein, *BBX4/COL3* (*CONSTANS LIKE-3*), has also been implicated in light regulated development (Datta et al., 2006). Unlike *BBX18-24* and *BBX32*, *BBX4/COL3* belongs to the Constans-like class of BBX proteins that is defined by the B-Box and the presence of the highly conserved Constans, Constans-Like, TOC1 (CCT) domain in the C-terminal region of the protein (Griffiths et al., 2003). *BBX4/COL3* is required for the proper integration of high fluence-rate red light, and does not appear to be necessary in responding to other wavelengths of light (Datta et al., 2006).

Nuclear Factor Y (*NF-Y*) transcription factors also have demonstrated roles in light responses, in particular the regulation of photosynthesis-related genes (Miyoshi et al., 2003; Warpeha et al., 2007; Stephenson et al., 2011). NF-Y transcription factors are comprised of three unrelated subunits, NF-YA, NF-YB

and NF-YC and bind to the core nucleotide sequence CCAAT (Mantovani, 1999; Dolfini et al., 2012). All three subunits are required in a complex to form the mature DNA binding transcription factor. While mammalian genomes have only one copy of each *NF-Y* subunit, each subunit has been vastly expanded into families in the angiosperm lineage (Siefers et al., 2009; Laloum et al., 2012). For example, the Arabidopsis genome encodes 10 *NF-YA*, 10 *NF-YB* and 10 *NF-YC*, allowing for 1000 unique possible NF-Y combinations.

Warpeha *et.al.* (2007) demonstrated a role for *NF-Ys* in response to very low fluence rate blue light in Arabidopsis. *NF-YA5* and *NF-YB9* mutant plants had impaired induction of *LIGHT HARVESTING CHLOROPHYLL A BINDING PROTEIN B 1.2* (*LHCB1.2*) in response to blue light stimulus. Although expression of *LHCB1.2* gene was impaired in *NF-Y* mutant lines, no morphological phenotypes were associated with these mutants. Prior to the Warpeha study, *NF-YB9* (also known as *LEC1*) was well characterized as a regulator of embryo development in plants. In a study to identify direct targets of NF-YB9 transcriptional control using chromatin immunoprecipitation, several light regulated genes were identified, supporting a role for *NF-YB9* in light signaling (Junker et al., 2012). Additionally, inducible overexpression of *NF-YB9* in Arabidopsis seedlings resulted in partial etiolation, implicating *NF-YB9* as a negative regulator of photomorphogenesis (Junker et al., 2012).

Orthologs of *NF-Ys* have been identified in rice (*OsNF-Y*) and wheat (*TaNF-Y*) and seem to share a similar function as those in Arabidopsis. Transcript expression analysis in wheat revealed that mRNA regulation of several *TaNF-YB* and *TaNF-YC* are correlated with that of photosynthetic genes (Stephenson et al., 2010, 2011). Additionally, over-expression of *TaNF-YB3* in wheat led to the increased expression of photosynthesis related genes, including *Lhcb* (Stephenson et al., 2011). In rice, three NF-YB subunits have been identified and appear to have overlapping roles in chloroplast biogenesis. Antisense or RNAi constructs targeted against *OsNF-YB2*, *OsNF-YB3* and *OsNF-YB4* in rice resulted in plants with reduced levels of chlorophyll, degenerate chloroplast and reduced expression of nuclear encoded photosynthetic genes (Miyoshi et al., 2003). These results suggest that *NF-Y* function in light signaling is conserved in the plant lineage.

There is mounting evidence that NF-Y transcription factors can physically interact with both the above-mentioned bZIP proteins and Constans-like BBX proteins to affect plant growth and development (Ben-Naim et al., 2006; Wenkel et al., 2006; Kumimoto et al., 2010). For example, it is well established that NF-Ys interact with CONSTANS (CO), the founding member of the CO-like family, to activate flowering in response to inductive photoperiods (Kumimoto et al., 2010). Genetic studies have also shown that a functional NF-Y is required for full CO activity. Additionally, NF-Ys can interact with numerous distantly related CO-like genes using yeast-2-hybrid (Wenkel et al., 2006; Kumimoto et al.,

2010). Although broad functional interactions between *NF-Y* and *CO-like* genes appear likely, examples outside of the flowering time module are still lacking. Conversely, interactions between bZIP proteins and NF-Y have been shown in diverse eukaryotic organism with occurrences in mammals, fungi and plants (Yoshida et al., 2000, 2001; Hortschansky et al., 2007; Yamamoto et al., 2009a). For example, bZIP/NF-Y interactions mediate endoplasmic reticulum stress responses in both plants and animals. Additionally, in plants bZIP/NF-Y interactions appear to be involved in abscisic acid (ABA) related signaling in both embryo development and seed germination (Yamamoto et al., 2009a).

Using yeast two hybrid assays, we previously demonstrated that NF-YC subunits could physically interact with HY5 as well as COL3. Here we show that, similar to *HY5* and *COL3*, *NF-Y* regulates light mediated phenotypes in both red and blue light. In particular, *NF-YC3*, *NF-YC4* and *NF-YC9* are necessary for proper red light signaling while only *NF-YC3* and *NF-YC9* are involved in blue light responses. Interestingly, these phenotypes imply that *NF-Ys* are positive regulators of photomorphogenesis, which is the opposite of what would be expected from over-expression results obtained with *LEC1/NF-YB9* mentioned above (Junker et al., 2012). Furthermore, we provide evidence that NF-YB3 is involved in blue light signaling. These data support the growing evidence that *NF-Y* can form diverse transcriptional complexes and play a key role in light regulated plant growth and development.

Results

nf-yb and *nf-yc* mutants show long hypocotyls under short day conditions.

We previously reported that NF-YC3, NF-YC4 and NF-YC9 have overlapping function in photoperiodic flowering. In addition, all three have been shown to physically interact with the light signaling genes HY5 and COL3 in yeast two-hybrid assays. A hallmark of photomorphogenesis is inhibition of hypocotyl length. Plants that have impaired light signaling often show alterations in hypocotyl elongation. For example, *HY5* is hyposensitive to light and shows a long hypocotyl phenotype in all tested light conditions (Ang and Deng, 1994; Oyama et al., 1997; Ang et al., 1998b), whereas *COL3* only shows elongated hypocotyls under short day and high fluence red light conditions (Datta et al., 2006). To test whether *NF-Y* play a role in light perception, we measured hypocotyl lengths of mutants for *nf-yc3*, *nf-yc4*, *nf-yc9*, and all possible mutant combinations therein under standard long day conditions (16hr light/8hr dark). Unlike *HY5*, no *nf-yc* mutant (singles or combinations) showed a hypocotyl elongation phenotype under long day conditions (data not shown). Under short day conditions (8hrs light/16hrs dark) both the *nf-yc3/c9* double and *nf-yc3/c4/c9 triple* mutant (here after *nf-yc-triple*) had longer hypocotyls than wild-type controls (Figure 4.1A,C). As previously shown for photoperiodic flowering (Kumimoto et al., 2010), the *nf-yc3/c9* double mutant is much weaker than the *nf-yc-triple*, implying overlapping functionality of *NF-YC3*, *NF-YC4* and *NF-YC9*.

Both NF-YB2 and NF-YB3 physically interact with NF-YC3 and NF-YC4 *in-vivo* to regulate photoperiodic flowering, thus we hypothesized they would also be involved in light perception (Kay et al., 1987; Kumimoto et al., 2010). Therefore, we extended this study to include *nf-yb2* and *nf-yb3* knockdown mutants and plants ubiquitously over-expressing *NF-YB2* and *NF-YB3* using the cauliflower mosaic virus 35S promoter (*35S:NF-YB2*, *35S:NF-YB3*) (Kay et al., 1987). Under long day conditions, there were no observable differences in hypocotyl lengths between the mutants and over expressers compared to controls (data not shown). Under short day conditions, the *nf-yb3* single mutants showed longer hypocotyls than controls (Figure 4.1B). The *nf-yb2* mutant showed no clear differences to controls while the *nf-yb2/b3* double mutant showed a nearly identical hypocotyl elongation phenotype to the *nf-yb3* single mutant.

NF-YB and NF-YC mutants show altered responses to Red and Blue light but not Far-Red light.

To investigate the roles of *NF-YB* and *NF*-YC genes in perception of light quality, we tested growth in three monochromatic light conditions, as well as in complete darkness. Plants were grown in continuous red (660nm), far red (730nm), and blue (450nm) light for 5 days and hypocotyl length measured. In addition to wild-type plants, controls for this experiment included *hy5* and *cop1-4* (*CONSTITUTIVE PHOTOMORPHOGENESIS 1*) mutants. *hy5* has long hypocotyls under all light conditions, while *cop1-4*, has short hypocotyls under

both light and dark conditions (Ang and Deng, 1994; Ang et al., 1998b; Osterlund et al., 1999).

Dark grown *nf-yc* single, double or triple mutant plants showed no growth differences compared to wild-type plants (Figure 4.2A). Under continuous red light, only the *nf-yc triple* mutant had significantly longer hypocotyls than controls at means of 9.0mm and 5.8mm, respectively (Figure 4.2B). Far-red light treatment of *nf-yc* mutant lines yielded no significant differences to wild type plants, although *hy5* mutants showed significantly longer hypocotyls (Figure 4.2C). When grown under blue light, both the *nf-yc3/c9* double and *nf-yc triple* mutants were more etiolated than controls (Figure 4.2D). These results agree with a previous study that implicated *NF-YC9* in blue light signaling (Warpeha et al., 2007). The *nf-yc3/c9* double mutant and the *nf-yc triple* hypocotyl length did not differ statistically under blue light, suggesting that *NF-YC4* does not play a role in blue light signaling.

Results for the *nf-yb* mutants were similar to those obtained for *nf-yc* mutants, although the phenotypes were generally weaker. When grown in darkness or far-red light, *nf-yb* mutants and *NF-YB* over-expression lines showed no differences to controls (Figure 4.3A,C). The *nf-yb2/b3* double mutant had slightly longer hypocotyls than controls under red light conditions (Figure 4.3B). Although this phenotype was statistically significant in two out of three experiments, the differences were very minor in comparison to the phenotype

observed with the *nf-yc triple*. This may indicate that other *NF-YB* have over-lapping functionality with *NF-YB2/B3* and higher order mutants will be necessary to observe robust phenotypes. When grown in blue light, the results for *NF-YB* mutants were similar to those found under short day conditions. The *nf-yb3-2* mutant had consistently longer hypocotyls than control while *nf-yb2-1* showed no phenotype (Figure 4.3D). Unlike the short day experiment, *nf-yb2/b3* mutant hypocotyls were not longer than controls under blue light. This result is interesting because the *nf-yb2/b3* double was always slightly shorter than *nf-yb3-2* mutants under short day conditions. Plants overexpressing *NF-YB2* or *NF-YB3* had consistently shorter hypocotyls under blue light, whereas these lines show no differences in photomorphogenic growth in any other light conditions.

Discussion

NF-Y transcription factors have many roles in plants but their functions are not well understood. Here we provide evidence that several *NF-Y* transcription factors are required for the proper integration of light quality signals. Under red light (660nm), *NF-YC3*, *NF-YC4* and *NF-YC9* have overlapping functionality. However, in blue light (450nm), only *NF-YC3* and *NF-YC9* have signaling functions while *NF-YC4* appears to have no function. This is similar to our previous results showing that *NF-YC3* and *NF-YC9* have over-lapping roles in ABA mediated seed germination while *NF-YC4* showed the opposing phenotype. Although *NF-YC4* has no observable role in blue light signaling, this

may be masked by the activity of its close paralog *NF-YC1*. Plants doubly mutated for NF-YC1 and NF-YC4 will be necessary to test this hypothesis.

NF-YB9 (LEC1) was recently shown to repress photomorphogenesis (Junker et al., 2012). This is the opposite of *NF-YC3* and *NF-YC9* role in inducing photomorphogenesis; supporting the above hypothesis that NF-Y can form complexes with distinct and opposing functions in light signaling. Therefore, one hypothesis is that NF-YC4 and NF-YC1 can form complexes with NF-YB9 to repress photomorphogenesis while NF-YC3 and NF-YC9 form complexes that promote photomorphogenesis. In two additional cases, *NF-Y* subunits also demonstrate opposing phenotypes for a given response. We previously observed that *NF-YC4* promotes germination, whereas *NF-YC3* and *NF-YC9* inhibit germination. In photoperiodic flowering, over-expression of *NF-YB2* and *NF-YB3* both induce flowering while over-expression of *NF-YB1* represses the floral transition (Wenkel et al., 2006; Kumimoto et al., 2008). In the *NF-Y* family, opposing functionality of closely related proteins may be common and the mechanisms underlying this observation warrant further investigation.

In plants, *NF-Y* gene families are greatly expanded compared to other kingdoms. One hypothesis for the expansion could be that *NF-Y* temper functional out-puts through the formation of competing positive and negative transcriptional complexes. Examples of this type of the “gas-and-break” gene regulation to attenuate responses to light are widespread in the literature (Jiao

et al., 2007). In addition to the composition of the NF-Y trimer, other transcriptional regulators appear to interact with NF-Y. Previous to this study we demonstrated that both COL3 and HY5 could interact with NF-YC subunits. These higher order transcriptional complexes add to the ability of the plant to integrate multiple signals at a single locus. The fact that *35S:NF-YB2* and *35S:NF-YB3* only show shorter hypocotyls under blue light suggests that a blue light activated partner is necessary for full function of *NF-YB2* and *NF-YB3*. There is precedent for this idea in the NF-Y/CO interaction. Under long day conditions, where CO is stable, both *35S:NF-YB2* and *35S:NF-YB3* can induce premature flowering. In contrast, neither *NF-YB* can induce premature flowering under conditions where CO is absent or severely reduced in abundance - e.g., short day conditions (Kumimoto et al., 2008) or in a *co* mutant background (Tiwari et al., 2012). Thus, in this scenario, NF-Y complexes, or at least a subset, do not act alone as transcriptional activators but work as partners with other proteins. Protein interaction assays and other methods of identifying these partners are essential next steps in the evolution of this field.

Although *NF-Y* are necessary for proper integration of light quality signals, direct targets of *NF-Y* transcription factors remain elusive. In addition, the exact composition of any NF-Y trimer *in planta* remains to be determined. Extended studies that include higher order mutants of these transcription factors NF-Y subunits are necessary to understanding how plants integrate multiple light signals into developmental outcomes.

Materials and Methods

Plant material and growth

All plants in this study were of the Col-0 ecotype. Hypocotyl elongation under short day and long day conditions were performed in Conviron (model ATC13) growth chamber at 22°C at 100 μmoles^{1}m^{-2}s^{-1} fluence rate of white light. Monochromatic light was generated-using LEDs from Illumitex (part #'s EC2406AD45-UL106D, EC2406AD66-UL106D, EC2406AD73-UL106D). Hypotcotyl growth experiments were performed in a custom built chamber designed to maintain ambient temperature. Seeds were surface sterilized and plated on Gamborgs B5 media. Seeds were stratified for 48 hours at 4°C in complete darkness. Plants were grown for 5 days in the appropriate conditions then each plate was imaged and hypocotyl length measured using ImageJ (Abramoff et al., 2004). All *NF-YB*, *NF-YC* mutants have been previously described (Kumimoto et al., 2008; Kumimoto et al., 2010; Cao et al., 2011). Both *hy5* (SALK_096651) and *cop1-4* have also been previously described (McNellis et al., 1994; Chen et al., 2008)

Figures and Tables

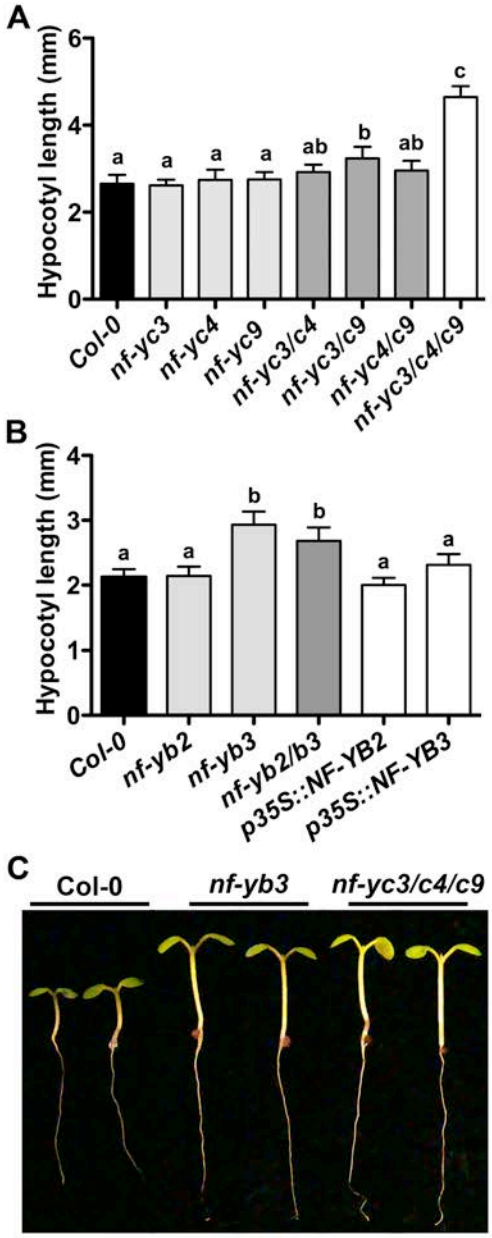


Figure 4.1. *NF-YB3*, *NF-YC3*, *NF-YC4*, and *NF-YC9* are negative regulators of hypocotyl elongation in short days. Hypocotyl length was recorded after 6 days of growth under short day conditions. A) *nf-yc3*, *nf-yc4* and *nf-yc9* single, double and triple mutants, B) *nf-yb2* and *nf-yb3* single and double mutants, *p35S::NF-YB2* and *p35S::NF-YB3*, C) Image of *nf-yb3* and *nf-yc triple* mutant phenotypes. Letters in panels A-B refer to significant differences for all pairwise comparisons using the Bonferroni multiple comparison procedure ($p<0.05$) after ANOVA ($p<0.0001$ for both experiments) - bars with the same letter indicate non-significant differences ($n \geq 11$). Measures of significance for the differences in panel B varied between experiments

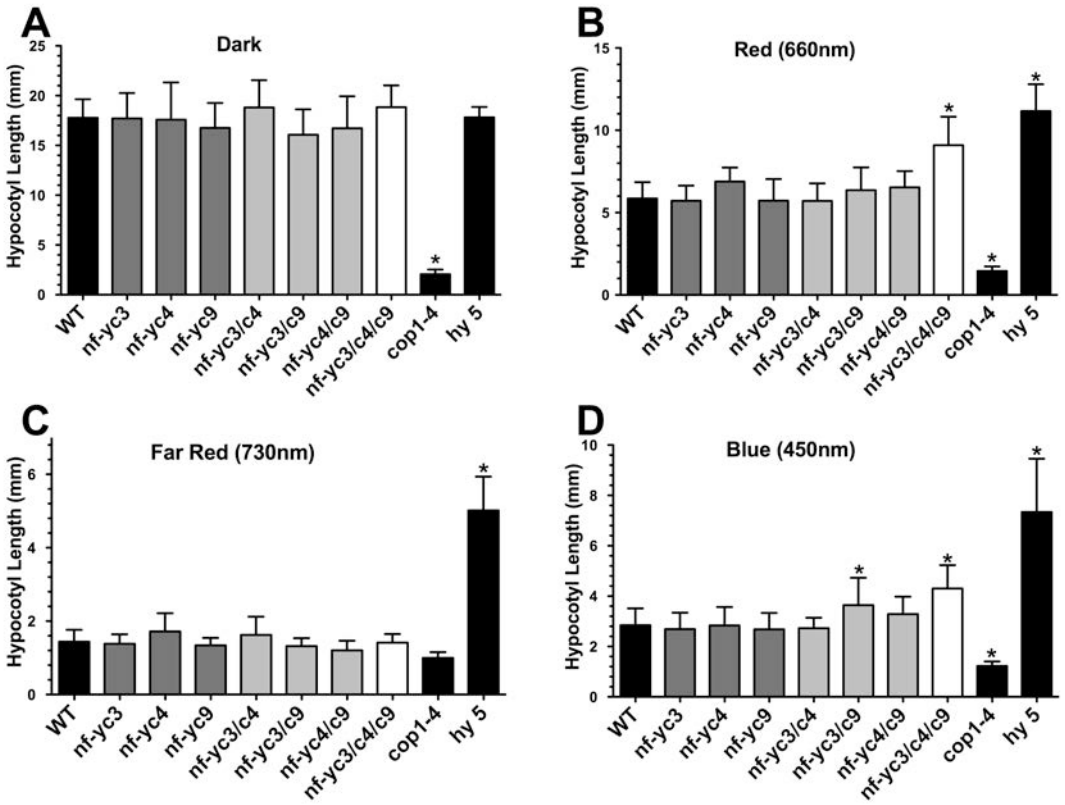


Figure 4.2 NF-YC3, NF-YC9 are positive regulators of photomorphogenesis under Red and Blue light. NF-YC4 is a positive regulator of photomorphogenesis under Red light only. Hypocotyl length was recorded after 5 days of growth under continuous monochromatic light conditions for *nf-yc3*, *nf-yc4* and *nf-yc9* single, double and triple mutants in A) Dark B) Red light (660nm), C) Far Red light (730nm), Blue light (450nm). Stars refer to significant differences compared to wild type using the Bonferroni multiple comparison procedure ($p<0.05$) after ANOVA. *cop1-4* and *hy5* were added as treatment controls

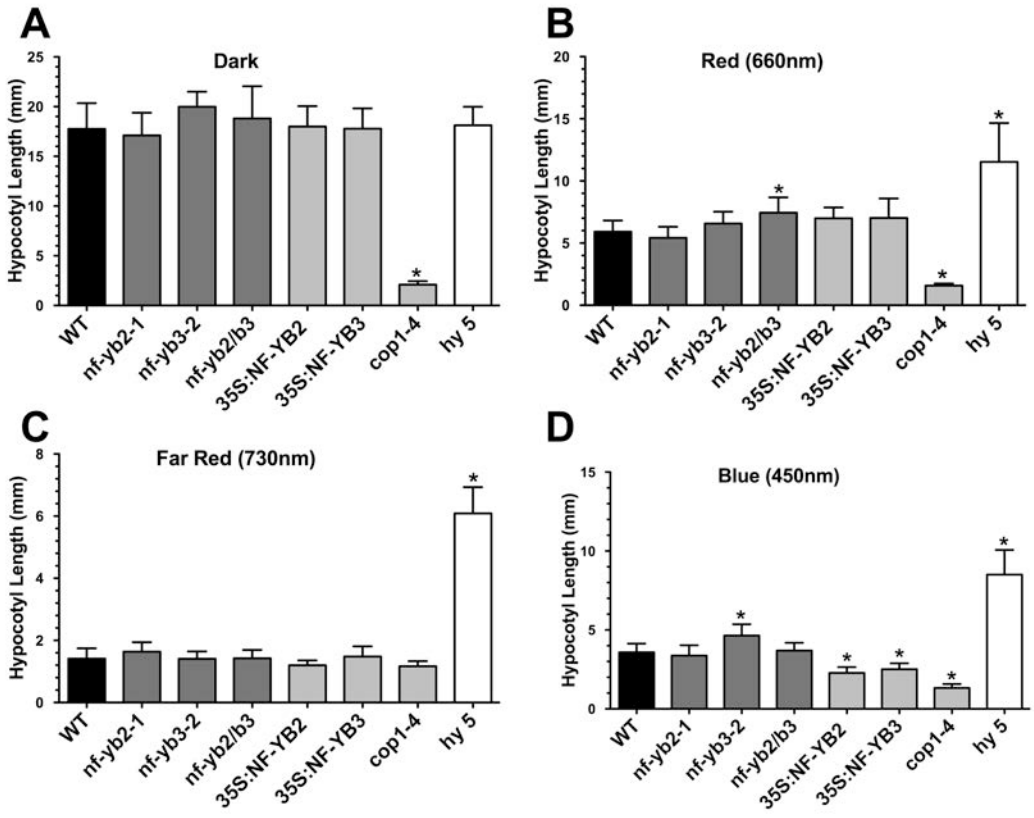


Figure 4.3 NF-YB3 is positive regulator of photomorphogenesis under Blue light. Hypocotyl length was recorded after 5 days of growth under continuous monochromatic light conditions for *nf-yb2*, *nf-yb3*, *nf-yb2/b3* double and 35S:NF-YB2, 35S:NF-YB3 in A) Dark B) Red light (660nm), C) Far Red light (730nm), Blue light (450nm). Star refers to significant differences compared to wild type using the Bonferroni multiple comparison procedure ($p<0.05$) after ANOVA. *cop1-4* and *hy5* were added as treatment controls.

Chapter 5: Transcriptome analysis of *CONSTANS* mutants reveals a potential role in salt tolerance

Introduction

The appropriate timing of flowering is vital for reproductive success in plants. Environmental signals such as light, temperature and nutrient availability all factor into the regulation of the floral transition. The ability to detect day-length, or photoperiod, is the primary cue for proper seasonal flowering in many plant species. Through genetic studies in *Arabidopsis thaliana*, a molecular model for sensing of photoperiod emerged with the gene *CONSTANS* (CO) as a central component (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002; Kobayashi and Weigel, 2007).

Arabidopsis maintains vegetative growth under short day conditions (<12hr light) and is induced to flower when grown under long day conditions (>12hr light). CO is required for sensing as well as activating flowering under inductive long day conditions. CO acts in the leaf phloem companion cells where it induces the transcription of *FLOWERING LOCUS T* (*FT*), the long sought mobile florigenic signal (Suarez-Lopez et al., 2001; An et al., 2004; Corbesier et al., 2007). While *FT* mRNA is produced in the vasculature, FT protein moves to the meristematic tissues where it activates floral organogenesis (Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Turck et al., 2008).

CO mRNA is circadian-clock regulated and peaks late in the 24-hour cycle. In contrast CO protein is light regulated, with CO protein being rapidly degraded in the dark (Suarez-Lopez et al., 2001; Valverde et al., 2004). These observations

led to the external coincidence model for photoperiod regulation of flowering. This model states that while CO mRNA is highest at the end of day in both short day and long day grown plants, CO protein can only accumulate to sufficient levels to activate *FT* when light coincides with peak mRNA levels.

CONSTANS is a founding member of the transcription factor family defined by the presence of a conserved Constans, Constans-Like, TOC1 (CCT) domain (Putterill et al., 1995; Robson et al., 2001; Griffiths et al., 2003). Although numerous experiments implicated CO as the direct activator of *FT*, there were no data demonstrating CO binding to the *FT* promoter until recently. Tiwari *et. al.* (2012) defined the promoter elements, CORE1 and CORE2, required for recruitment of CO to DNA in the *FT* promoter. Even though CO regulation of *FT* has been reasonably well-studied, other targets of CO are poorly defined.

Two previous studies were carried out to identify additional targets of CO. First dexamethasone inducible versions of CO were used to create a suppression subtraction hybridization cDNA library (Samach et al., 2000). This cDNA library was used to identify genes that were enriched due to the over-expression of *CO*. In addition to *FT*, two other genes were reported as being up regulated by CO. At the time of publication one gene was annotated as 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS10, AT1G62960) and was predicted to generate a precursor molecule for ethylene biosynthesis. Subsequent studies showed ACS10 does not have ACC synthase activity but is

an amidophosphoribosyltransferase with broad specificity for aspartate and aromatic amino acids (Yamagami et al., 2003). The second target, Δ^1-pyrroline-5-carboxylate synthase (*AtP5CS2, At3g55610*) is an enzyme that controls the rate limiting step of proline biosynthesis (Fabro et al., 2004). As well as being a necessary amino acid, plants accumulate proline in response to abiotic stresses such as drought or salt (Delauney et al., 1993). Proline is thought to act as an osmotic protectant for the stressed cells.

In addition to the suppression subtraction hybridization cDNA library, whole genome microarray studies were also performed. An experiment was performed comparing transcript profiles of multiple flowering time mutants, including *CO* and *FT*, after the transition from short day conditions to long day conditions (Schmid et al., 2003). Unfortunately, in this experiment the researchers used only tissue derived from dissected meristems to isolate mRNA. Subsequent studies revealed that *CO* is active in the leaves and not the meristem (An et al., 2004). Not surprisingly they discovered that the *FT* and *CO* mutant profiles were indistinguishable from one another. This is because CO activates *FT* expression in the leaf vasculature and FT protein subsequently translocates to the meristem to regulate transcription. Thus, inactivation of either *CO* or *FT* alters a very similar set of downstream targets in distal meristematic tissue.

Here we describe a microarray experiment comparing 10-day-old *co-10* mutant plants to wild-type controls grown under long day conditions. Whole above

ground tissue was harvested 15 hours after lights on, when CONSTANS protein levels are high and actively driving *FT* transcription (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002). Analysis of this data led to the hypothesis that *CO* plays a role in abiotic stress tolerance, specifically salt stress. Testing this hypothesis we show that plants over-expressing *CO* are sensitive to salt stress in plate based assays. In addition, it appears that *CO* is high in the hierarchy of a transcriptional cascade as transcription factors are over-represented in the down regulated gene set in the *co-10* mutant profile. Promoter site over-representation was also analyzed for this data set, uncovering new potential binding sites for CO. These results suggest that *CO* has roles in multiple signaling pathways, not only regulating flowering but also salt stress tolerance.

Results

Transcription factors are overrepresented in the CO microarray gene set.

Previously, we demonstrated that CO required NUCLEAR FACTOR Y (NF-Y) transcription factors to fully activate *FT* (Kumimoto et al., 2010). That led to the hypothesis that NF-Y and CO would regulate many of the same genes. To test this hypothesis, we ran Affymetrix ATH1 microarray experiments on late flowering *nf-yc3/c4/c9* triple and *co-10* mutants, comparing both to wild-type controls. Surprisingly, only *FT* appeared to be commonly regulated in this microarray study. To extend this study we reexamined the *co-10* microarray using the freely available ROBIN software package (Lohse et al., 2010). Gene lists containing significantly up and down regulated genes were produced using

RMA chip normalization and the default Benjamini Hochberg false discovery rate correction (p-value ≤ 0.05) . In total, 134 genes were statistically significantly down regulated and 156 up regulated. Because CO has been shown to be a transcriptional activator (Ben-Naim et al., 2006; Tiwari et al., 2010), we initially focused on the down regulated gene set.

Of the 134 down regulated genes, 41 were down regulated two-fold or greater (Table 5.1). The fact that both *CO* and *FT* were down regulated offered an initial quality control check for the microarray. Two highly down regulated genes were key enzymes regulating the phytohormone abscisic acid (ABA) and ethylene metabolism, Cytochrome P450 family member 707-A3 (CYP707A3) and ACC synthase 6 (ACS6), respectively. CYP707A3 hydroxylates the 8'-C of ABA, abolishing its activity (Kushiro et al., 2004). ABA is a key hormone in the regulation of abiotic stress tolerance (Cutler et al., 2010). Of interest is the fact that CYP707A3 is natively expressed in the same tissue as CO - the vasculature of leaves (Okamoto et al., 2008). This makes CYP707A3 an attractive target for further investigation as a possible direct target of CO transcriptional activation. ACS6 catalyzes a key step in ethylene biosynthesis, the conversion of S-adenosyl-methionine to 1-aminocyclopropane-1-carboxylic acid (Yamagami et al., 2003). Ethylene has a number of function in plants including root development, fruit ripening, resistance to abiotic and biotic stresses (Lin et al., 2009). ACS6 has general expression throughout the plant and is induced by hypoxia stress (Vandenbussche et al., 2003)

The most striking result in the down regulated gene set is the number of transcription factors present. Nineteen of the 41 genes two-fold or greater down regulated genes encode transcription factors. For all 134 down regulated genes (i.e., all statically significantly down regulated genes regardless of fold change), gene ontology (GO) category representation analysis was performed using the online Database for Annotation, Visualization and Integrated Discovery application (Huang da et al., 2009a; Huang da et al., 2009b). Table 5.2 shows all GO categories with a Bonferoni corrected p.value <0.05. This analysis showed that transcription factors are nearly 4 fold over-represented in the gene list. In addition, categories related to hormone signaling are over-represented. In particular, the ethylene-signaling pathway appears to be affected.

Examination of the up regulated gene list yielded only 26 genes that were changed 2 fold or greater (Table 5.3). Two of the 11 beta-1,3-glucanase (*BG2* and *BG3*) genes are among the most differentially regulated genes. Both BG2 and BG3 are predicted to have cellulase activity. Three other predicted cell wall metabolism genes are also represented in the 2-fold up regulated list (PECTIN METHYLESTERASE 41, PUTATIVE CHITINASE, XYLOGLUCOSYL TRANSFERASE 33). This brings up the possibility that CO has an unforeseen role in cell wall metabolism. *BG2* is also annotated as *PATHOGENESIS-RELATED GENE 2* (*PR2*) and two other *PR* genes were also up regulated (*PR1*, *PR5*), implying an augmented biotic stress defense response.

Over-represented GO terms for the up regulated gene list fell into two main categories, kinase activity and nucleotide binding (Table 5.4). Reexamination of the 2-fold or greater gene list does reveal two receptor kinases as well as MAP kinase 11 (MPK11). MPK11 has a known role in response to pathogens(Bethke et al., 2012), reinforcing the possible role of CO in biotic stress responses. In addition, due to the cell wall metabolism genes mentioned above, terms related to the cellular component cell wall were 3.5 fold over-represented in this gene list.

Promoter Element Analysis.

Recently, CO was reported to bind specifically to the CORE1 or CORE2 sequences (*TGTG$_{N2-3}$ATG*) in the promoter of *FT* (Tiwari et al., 2010). We extended our analysis of the *co-10* microarray by searching for instances of the CORE1 or CORE2 elements in the upstream 500bp of the down regulated gene set. Sixteen of the 134 (~12%) down regulated genes contain at least one CORE1 or CORE2 element. Using the bioinformatics tool PATMATCH on the TAIR website we identified that there are 4,613 instances of either CORE1 or CORE2 500bp upstream of the 33,602 expressed sequences (~14%). Chi squared analysis of the proportion of CORE motifs in the genome versus in the *co-10* microarray confirmed that the CORE elements are not over-represented in promoters of the down regulated genes.

To search for over-represented promoter motifs in the mis-regulated gene sets we used the online analysis suite ATHENA (O'Connor et al., 2005). Within the 134 significantly down (p.value $<10^{-4}$) regulated genes, the ABA Responsive Element-Like (ABRE) promoter element (BACGTGKM) was ~2 fold enriched (28% in our set versus 12% in the genome). The ABRE is found in the promoters of many ABA regulated genes. Because ABA is the major stress hormone in plants over representation of this element further supports a role for CO in abiotic stress responses.

In addition to searching for known promoter motifs using Athena, we used Multiple EM for Motif Elicitation (MEME) to uncover new possible promoter elements. Unlike ATHENA that relies on *a priori* knowledge of promoter motifs, MEME uses a hidden Markov algorithm to generate conserved motifs between sequences *de novo* (Bailey et al., 2006). MEME analysis was performed on 500bp of sequence up-stream of the transcriptional start site for genes -1.5 fold or more down regulated. This analysis yielded two low E-value motifs (FIG 1 A,C). Each discovered motif was then queried against databases containing known promoter motifs for similarity using the software TOMTOM (Gupta et al., 2007). Motif 1 (Figure 5.1A) showed no similarity to known promoter motifs using TOMTOM search. Similarity search of Motif 2 (Figure 5.1C) revealed significant similarity to the previously reported binding sites of bHLH transcription factors in mouse. This motif also contains the ABRE core sequence of *ACGT*. Analyses of the positions of both discovered elements

clearly showed that Motif 1 was biased towards the transcriptional start site - greater than half of the Motif 1 elements were found in the first 100bp of the promoter (Figure 5.1B). Motif 2 was more evenly distributed across the first 500bp of promoter (Figure 5.1D).

MEME analysis of the promoter region of the up regulated gene set did not yield any low E-value motifs above background. Athena analysis of the up regulated promoter sets did show a slight yet statistically significant enrichment for the W-box promoter element (66% vs 42% or approx. 1.5 fold). The W-box (TTGACY) is bound by WRKY transcription factors and is primarily related to biotic stress responses, specifically induction by salicylic acid. There is one WRKY TF in the up regulated gene list, *WRKY25* (Table 5.3). Interestingly, *WRKY25* has a demonstrated role in salt stress tolerance (Jiang and Deyholos, 2009).

CO over-expression plants are sensitive to salt stress.

Several lines of evidence from the analysis of the CO mutant microarray led to the hypothesis that CO plays a role in salt stress tolerance. First, genes related to ABA signaling are down regulated in the mutant background. ABA is the central phytohormone related to abiotic stress responses. Salt is a well-known activator of the ABA stress responsive pathway. Promoter analysis of the down regulated gene set showed an over-representation of the abiotic stress responsive ABRE. In addition, previous studies have shown that *CO* is down regulated upon salt stress (Li et al., 2007). Three TFs in the down regulated

gene set are transcriptionally responsive to salt stress (AZF1, STZ1 and SZF). AZF1 and STZ1 are phylogenetically related and mutants affect salt stress tolerance (Sakamoto et al., 2004; Mittler et al., 2006). Although the up regulated gene list primarily contained genes with predicted associations with biotic stresses, the TF WRKY25 also has a demonstrated role in NaCl tolerance.

To test the hypothesis that CO plays a role in salt stress tolerance, we examined *co-10* mutant's ability to survive salt stress. Wild-type and *co-10* mutants plants were germinated and grown on Gamborgs B5 media for 4 days then transferred to media supplemented with increasing concentrations of NaCl. Plant survival was assayed every 24 hours for 4 days. *CO* mutants showed no clear differences to control plants when grown on any concentration of NaCl (Figure 5.2).

We extended this study to include plants over-expressing *CO*. Two independent transgenic lines of *CO* driven by the cauliflower mosaic virus 35S promoter were assayed for survival in response to NaCl. Both *CO* overexpressing lines were much more sensitive to all concentrations of NaCl. This was particularly apparent on 150mM NaCl at 3 days post incubation. Wild-type plants have no difficulty surviving and growing on 150mM NaCl as nearly 100% survive. This is in contrast to *35S:CO* lines that have between 20-60% survival rates.

Discussion

The regulation of CO mRNA and protein are key to the control of photoperiodic flowering responses. Although the CO affect on flowering has been well characterized, genome level studies of CO were lacking. Microarray analysis of gene expression of *CO* mutants revealed a possible role in abiotic stress tolerance, specifically salt tolerance. Salt is an osmotic stress that retards growth and delays flowering in Arabidopsis (Achard et al., 2006; Li et al., 2007).

Although the affect of *CO* on stress related flowering has been studied, affects of *CO* on stress tolerance are poorly reported. Here we demonstrated that over-expression of *CO* is detrimental to plant survival during salt stress. Because *CO* mRNA levels are natively suppressed by salt (Li et al., 2007), it appears likely that *CO* activity and/or reproductive growth affects the plants' ability to deal with increasing levels of salt. While the *CO* over-expression lines have clear NaCl stress phenotypes, the same cannot be said for the *co-10* loss of function mutants. CO belongs to a large family of highly related proteins and functional redundancy may be obscuring *CO* functionality in salt stress. Although *CO* mutants showed no salt stress phenotypes; cold stress tolerance has been reported (Yoo et al., 2007), reinforcing a native role for *CO* in abiotic stress tolerance.

General examination of the down regulated gene set in *co-10* mutants revealed an over-representation of ethylene response pathway components. Analysis of

microarray data performed on salt stressed wild-type Arabidopsis plants showed an up-regulation of ethylene related genes (Ma et al., 2006). Among these genes *ACS6* appears specific to salt stress and *ACS6* was among the most strongly down regulated genes in the *co-10* microarray set. Two TFs up regulated by salt are also down regulated in the *co-10* mutant, *ZAT10* and *WRKY53*. Thus, both phenotypic and microarray data support the hypothesis that CO can integrate flowering and abiotic stress signals.

CO protein accumulation can potentially be regulated by abiotic stress. CO physically interacts with the RING finger–containing E3 ubiquitin ligase HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1 (HOS1) (Lazaro et al., 2012). HOS1 has demonstrated roles in the cold acclimation responses of Arabidopsis(Lee et al., 2001; Dong et al., 2006). These data are consistent with the cold tolerance seen in CO mutant alleles (Yoo et al., 2007). In addition, HOS1 mutants are early flowering reflecting the higher levels of CO protein. Although a physical interaction between CO and HOS1 has been demonstrated, CO protein accumulation in response to stress has not been directly measured. This is clearly an avenue for further investigation.

Analysis of the promoter elements within the *co-10* down regulated gene set yielded a previously unknown motif as well as a binding site for bHLH TFs (Figure 5.1). Because so many TFs are present within the down regulated gene set,, it remains to be determined whether CO can bind these elements or

if their over-representation is the result of down stream, indirect activation. Further investigation using chromatin immunoprecipitation as well as *in vitro* DNA binding assays will be necessary to fully characterize the elements directly bound by CO. The previously described binding motifs for CO did not appear in the mis-regulated gene set more that expected by random chance. This could be due to the fact that expression for genes containing those motifs are attenuated in the steady state system of the null mutant background. The microarray results only represent a single time point; further study based on a time course would be more informative. Again, inducible versions of *CO* over-expression would be more ideal for assaying direct targets of *CO* moving forward.

CO has been well studied as an inducer of photoperiodic flowering. This and other studies are starting to provide evidence that CO not only integrate light signals but abiotic stress into the flowering pathway. The cross regulation of CO by multiple signaling pathways allows for the fine-tuning of flowering and stress responses. The ability to fine tune developmental responses to the surrounding environment provides an adaptive advantage and lays the ground work for success of the next generation.

Materials and Methods

Microarray Analysis

All microarray data was collected and recorded in compliance with MIAME (Brazma et al., 2001). Unprocessed microarray data (.cel and .chp files) and

detailed experimental conditions are publicly available at the NASCarrays website (http://Affymetrix.arabidopsis.info/narrays/experimentpage.pl?experimentid=440) using the experiment identifier NASCARRAYS-440. Plants for microarray analysis were grown for 10 days under standard long day conditions; above ground tissue was harvested 14 hours after chamber lights on. For each plant genotype - Col-0 parental control, and *co-10* - total RNA was collected from 10 plants/biological for triplicate replicates using the Qiagen RNeasy Plant Mini Kit. Total RNA was sent to the Nottingham Arabidopsis Stock Center (NASC) for quality control and hybridization to Affymetrix ATH-1 microarray chips (www.Affymetrix.com). Resulting data were analyzed with ROBIN software package with the following settings: Normalization settings for chip quality control: normalization method - RMA, P-value correction method - BH, analysis strategy - Limma. Normalization for main analysis included: normalization method - RMA, P-value correction method - BH, Multiple testing strategy - nestedF. These values represent the default settings for analysis in the ROBIN software package. Resulting gene list were filtered for genes misregulated with a BH corrected p-value ≤ 0.05. These gene lists were used for all down stream analysis (GO category, MEME, Athena).

Gene Ontogeny (GO) Category over-representation analysis

GO category over-representation analysis was performed using the online application the Database for Annotation, Visualization and Integrated Discovery DAVID, (Huang da et al., 2009a; Huang da et al., 2009b). The TAIR_ID's

(Arabidopsis Genome Initiative ID, AGI) of all statistically significant up or down regulated genes, regardless of fold change, were fed to the DAVID software package. The background for statistical analysis was set as Affymetirx ATH1-121501 genome array. All GO categories with a Bonferoni corrected p.value <0.05 were collected

Promoters Motif elicitation

MEME de-novo motif discovery was performed on -500bp of upstream sequence of the misregulated ≤ +/- 1.5 fold genes. Sequences were obtained using the bulk data retrieval tool at The Arabidopsis Information Resource (Swarbreck et al., 2008). FASTA files containing the upstream DNA sequences were fed to the MEME program and analyzed using the following parameters: motif width between 5 and 10bp, any number of repetitions of motifs, and search for up to 6 motifs. All other options were left as default (http://meme.nbcr.net/meme4_5_0/intro.html, (Bailey et al., 2006)). Motifs discovered through MEME analysis were then compared against known transcription factor binding sites from Jasper, Transfac and Uniprobe using the TOMTOM motif comparison tool (Gupta et al., 2007). Positions of *MOTIF1 and MOTIF2* relative to the transcriptional start were adapted from the MEME analysis. The same misregulated gene lists were input into the Athena analysis suite using a 500 max bp upstream cutoff and, otherwise, default settings (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl, (O'Connor et al., 2005))..

Plant Growth and Transgenic lines.

All plants grown for this study were of Col-0 accession. Constans (AGI AT5G15840) 35S:CO line #1 was previously analyzed [Kumimoto et al 2010]. Constans OEX line #2 was generated using the destination vector pEARLYGATE203 (TAIR stock CD3-689) and pENTR223 containing CO cDNA (TAIR stock GC105432). Recombination reactions between pENTR223 and pEARLYGATE203 were performed using LR clonase II as per manufacturers instructions (Invitrogen). In this construct CO is driven by the 35S promoter and has a N-terminal translation fusion to the MYC epitope tag. Quantitative qPCR shows that both 35S:CO lines are highly over-expressed (Fold changes). In addition both lines are early flowering (data not shown). The ***co-10*** allele (also published as *co-SAIL*) is a TDNA insert 342 bp down stream of the translational start, was previously described (Laubinger et al., 2006). Seeds for the *co-10* allele were a gift from George Coupland.

After 3 days of cold stratification seeds for salt stress survival assay were germinated in 24hrs of light at 22C on Gamborgs B5 containing media. After 4 days of growth seedlings were transferred to either Gamborgs B5 media as control or Gamborgs B5 containing 100mM, 150mM or 200mM NaCl. Survival was scored every 24 hours for 4 days after transfer. Plants with bleached a meristematic region were scored as dead, whereas green meristematic regions were counted as alive.

Figures and Tables

Table 5.1. Genes Down Regulated 2 fold or greater in co-10 mutant. AGI = Arabidopsis Genome Initiative number.

AGI	Fold change	p.Val	Annotation
At2g20670	-5.7	3.1E-04	Protein of unknown function (DUF506)
At5g45340	-4.4	3.1E-04	CYP707A3, Cytochrome P450, family 707, subfamily A, polypeptide 3
At1g80440	-4.0	3.1E-04	Galactose oxidase/kelch repeat superfamily protein
At4g11280	-3.9	5.5E-04	ACS6, 1-aminocyclopropane-1-carboxylic acid (acc) synthase 6
At2g30040	-3.5	9.1E-04	MAPKKK14__mitogen-activated protein kinase kinase kinase 14
At1g21910	-3.3	3.1E-04	DREB26, transcription factor family protein
At5g61600	-3.1	7.1E-04	ERF104__ethylene response factor 104, transcription factor
At4g27410	-3.1	4.0E-03	RD26 NAC (No Apical Meristem) domain transcriptional regulator
At2g41640	-3.0	1.8E-02	Glycosyltransferase family 61 protein
At3g19680	-2.9	7.7E-04	Protein of unknown function (DUF1005)
At4g17460	-2.7	6.6E-04	HAT1 Homeobox-leucine zipper protein 4 (HB-4) / HD-ZIP
At4g37260	-2.7	9.1E-04	MYB73, myb domain protein 73
At1g77640	-2.7	1.7E-02	DREB subfamily A-5 of ERF/AP2, transcription factor
At4g29780	-2.5	1.7E-02	unknown protein
At4g27280	-2.5	8.8E-04	Calcium-binding EF-hand
At3g50060	-2.5	2.1E-03	MYB77__myb domain protein 77
At1g27730	-2.5	1.7E-02	STZ_ZAT10__salt tolerance zinc finger
At5g15840	-2.5	3.1E-04	CO_FG_B-box type zinc finger protein with CCT domain
At1g33760	-2.4	4.5E-03	DREB subfamily A-4 of ERF/AP2
At5g52050	-2.4	4.0E-03	MATE efflux family protein
At5g67300	-2.4	3.5E-03	ATMYB44_ATMYBR1_MYB44_MYBR1__myb domain protein r1
At1g04770	-2.3	4.9E-03	Tetratricopeptide repeat (TPR)-like superfamily protein
At4g23810	-2.3	2.6E-02	ATWRKY53_WRKY53__WRKY family transcription factor
At5g51190	-2.3	7.3E-03	ERF (ethylene response factor) subfamily B-3 of ERF/AP2,
At1g76600	-2.2	1.5E-03	unknown protein
At1g13260	-2.2	4.9E-03	EDF4_RAV1__related to ABI3/VP1 1
At3g44990	-2.2	1.9E-02	XTH31_XTR8__xyloglucan endo-transglycosylase-related 8
At3g55980	-2.1	1.3E-02	ATSZF1 SZF1 salt-inducible zinc finger 1
At1g07050	-2.1	7.7E-03	CCT motif family protein
At2g40000	-2.1	4.8E-02	HSPRO2 ortholog of sugar beet HS1 PRO-1 2
At4g37240	-2.1	2.5E-03	unknown protein
At5g67450	-2.1	2.8E-02	AZF1_ZF1__zinc-finger protein 1
At5g15950	-2.1	3.1E-04	CPuORF10 conserved peptide upstream open reading frame 10
At5g42200	-2.0	2.6E-03	RING/U-box superfamily protein
At3g11410	-2.0	7.7E-04	AHG3, PP2CA, protein phosphatase 2CA
At4g28290	-2.0	8.4E-03	unknown protein
At1g72920	-2.0	2.4E-02	Toll-Interleukin-Resistance (TIR) domain family protein
At1g25560	-2.0	4.8E-03	EDF1_TEM1__AP2/B3 transcription factor family protein
At4g01950	-2.0	2.6E-02	ATGPAT3_GPAT3__glycerol-3-phosphate acyltransferase 3
At4g24570	-2.0	1.6E-02	DIC2__dicarboxylate carrier 2
At1g65480	-2.0	2.9E-03	FT, (phosphatidylethanolamine-binding protein)

Table 5.2. GO categories over-represented in *co-10* down regulated gene set

GO Term	GO Annotation	Fold	p.val
Molecular Function	GO:0003700 transcription factor activity	3.7	5.78E-10
Molecular Function	GO:0030528 transcription regulator activity	3.5	6.73E-10
Biological Process	GO:0010033 response to organic substance	3.7	1.02E-07
Biological Process	GO:0009723 response to ethylene stimulus	8.4	6.76E-07
Biological Process	GO:0045449 regulation of transcription	2.7	5.44E-06
Biological Process	GO:0009719 response to endogenous stimulus	3.7	5.67E-06
Biological Process	GO:0009725 response to hormone stimulus	3.9	7.49E-06
Molecular Function	GO:0003677 DNA binding	2.6	1.17E-05
Biological Process	GO:0009873 ethylene mediated signaling pathway	9.6	6.26E-05
Biological Process	GO:0000160 two-component signal transduction system (phosphorelay)	8.1	7.45E-05
Biological Process	GO:0010200 response to chitin	11.3	7.52E-05
Biological Process	GO:0009743 response to carbohydrate stimulus	8.1	3.20E-04
Biological Process	GO:0032870 cellular response to hormone stimulus	5.0	4.28E-04
Biological Process	GO:0009755 hormone-mediated signaling	5.0	4.28E-04
Biological Process	GO:0006355 regulation of transcription, DNA-dependent	3.0	4.11E-03
Biological Process	GO:0051252 regulation of RNA metabolic process	3.0	4.52E-03
Biological Process	GO:0007242 intracellular signaling cascade	3.3	6.02E-03
Biological Process	GO:0006350 transcription	2.7	1.49E-02
Biological Process	GO:0009628 response to abiotic stimulus	2.6	1.70E-02
Molecular Function	GO:0016564 transcription repressor activity	14.7	4.58E-02

Table 5.3. Genes Up Regulated 2 Fold or Greater in *co-10* mutant. AGI = Arabidopsis Genome Initiative number

AGI	Fold change	p.Val	Annotation
AT5G24420	3.1	2.2E-02	PGL5__6-phosphogluconolactonase 5
AT2G24850	3.1	1.4E-02	TAT_TAT3__tyrosine aminotransferase 3
AT4G02330	2.6	3.7E-03	Plant invertase/pectin methylesterase inhibitor superfamily
AT2G18660	2.6	4.9E-03	PNP-A__plant natriuretic peptide A
AT2G43570	2.5	2.6E-02	CHI__chitinase, putative
AT3G57240	2.5	1.1E-02	BG3__beta-1,3-glucanase 3
AT1G75040	2.4	3.3E-03	PR-5_PR5__pathogenesis-related gene 5
AT5G04360	2.3	2.6E-03	ATLDA_ATPU1_LDA_PU1__limit dextrinase
AT4G02380	2.3	3.0E-03	AtLEA5_SAG21__senescence-associated gene 21
AT3G62150	2.3	6.6E-04	PGP21__P-glycoprotein 21
AT3G23120	2.2	4.9E-03	AtRLP37_RLP37__receptor like protein 37
AT4G14610	2.2	3.1E-04	pseudogene, disease resistance protein
AT1G19380	2.2	1.7E-02	Protein of unknown function (DUF1195)
AT5G60900	2.1	1.5E-03	RLK1__receptor-like protein kinase 1
AT5G25440	2.1	1.1E-03	Protein kinase superfamily protein
AT4G23150	2.1	1.6E-02	CRK7__cysteine-rich RLK (RECEPTOR-like protein kinase) 7
AT3G57260	2.1	3.7E-02	BG2_BGL2_PR-2_PR2__beta-1,3-glucanase 2
AT2G38240	2.1	4.4E-02	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase
AT2G24160	2.0	3.3E-03	pseudogene, leucine rich repeat protein family
AT2G30250	2.0	7.7E-03	ATWRKY25_WRKY25__WRKY DNA-binding protein 25
AT1G01560	2.0	2.8E-03	ATMPK11_MPK11__MAP kinase 11
AT4G14365	2.0	4.9E-03	XBAT34__XB3 ortholog 4 in Arabidopsis thaliana
AT5G22380	2.0	3.3E-02	anac090_NAC090__NAC domain containing protein 90
AT1G10550	2.0	1.5E-02	XET_XTH33__xyloglucan:xyloglucosyl transferase 33
AT3G51860	2.0	1.4E-02	ATCAX3_ATHCX1_CAX1-LIKE_CAX3__cation exchanger 3
AT2G14610	2.0	3.8E-02	ATPR1_PR 1_PR1__pathogenesis-related gene 1

Table 5.4. GO categories over-represented in *co-10* up regulated gene set

GO Term	GO Annotation	Fold	p.val
Molecular Function	GO:0004674 serine/threonine kinase activity	4.2	1.2E-06
Molecular Function	GO:0004672 protein kinase activity	3.8	2.2E-06
Biological Process	GO:0006468 protein amino acid phosphorylation	3.4	5.5E-05
Biological Process	GO:0016310 phosphorylation	3.1	2.3E-04
Biological Process	GO:0006793 phosphorus metabolic process	3.0	2.5E-04
Molecular Function	GO:0032559 adenyl ribonucleotide binding	2.4	3.7E-04
Molecular Function	GO:0030554 adenyl nucleotide binding	2.3	4.6E-04
Molecular Function	GO:0001883 purine nucleoside binding	2.3	4.6E-04
Molecular Function	GO:0001882 nucleoside binding	2.3	4.9E-04
Biological Process	GO:0006796 phosphate metabolic process	2.9	9.2E-04
Molecular Function	GO:0005524 ATP binding	2.2	2.9E-03
Molecular Function	GO:0032553 ribonucleotide binding	2.1	3.1E-03
Molecular Function	GO:0032555 purine ribonucleotide binding	2.1	3.1E-03
Molecular Function	GO:0017076 purine nucleotide binding	2.1	3.7E-03
Cellular Component	GO:0005618 cell wall	3.5	1.3E-02
Cellular Component	GO:0030312 external encapsulating structure	3.5	1.4E-02
Cellular Component	GO:0005886 plasma membrane	2.0	3.2E-02

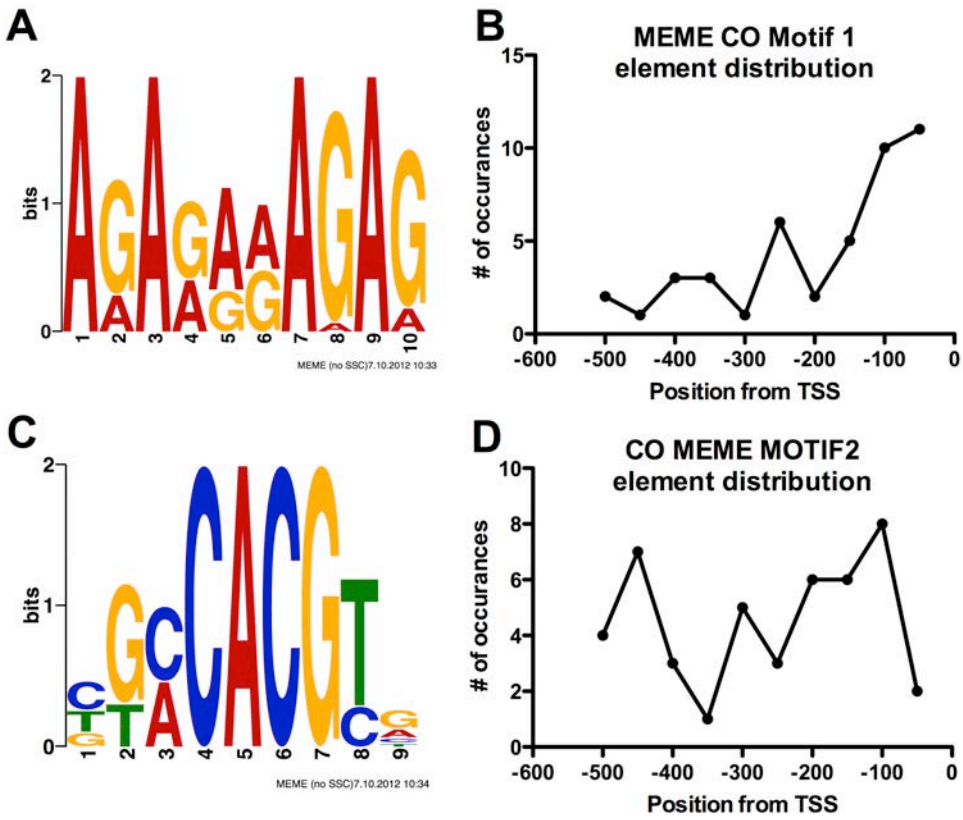


Figure 5.1 Discovery of promoter motifs within genes down regulated in *co-10*. Promoter sequences -500bp of TSS was analyzed using MEME. Sequence Logos of low E valule motifs generated by MEME search **A)** Motif 1, **C)** Motif 2. Positional distribution of MEME motifs within the promoter relative to TSS (Transcriptional Start Site) for **B)** Motif 1 **D)** Motif 2

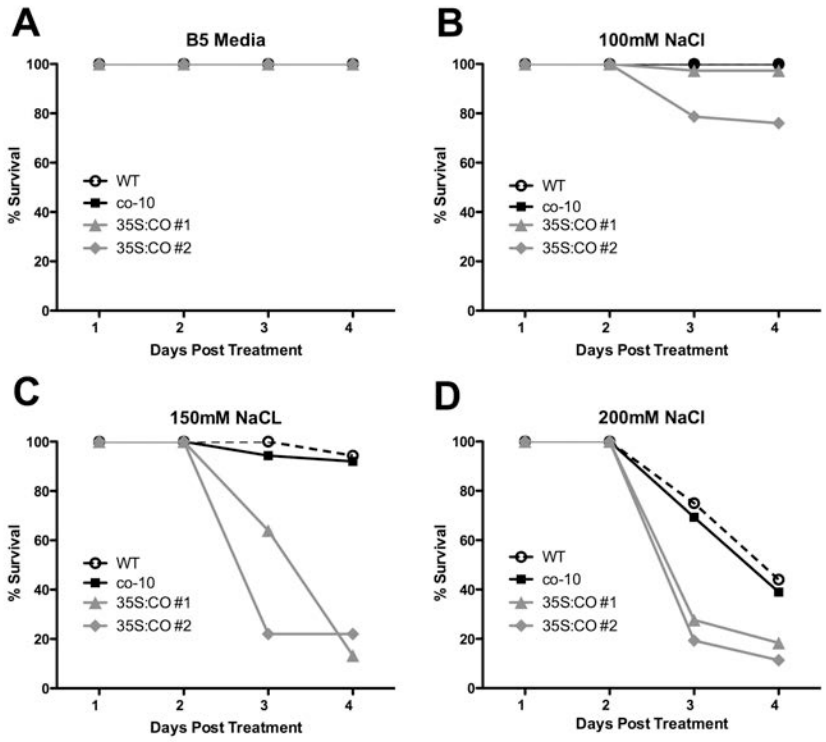


Figure 5.2 Constans over-expression plants are sensitive to salt.
Seedlings were germinated and grown on Gamborgs B5 basal media and transferred to plates supplemented with **A)** B5 **B)** 100mM NaCl **C)** 150mM **D)** 200mM NaCl. Survival was assayed every day for 4 days post treatment

Chapter 5: Conclusion

Conclusions and Future Directions.

The primary goal of my research was to extend the understanding of how plants respond and adapt to the environment around them. The focus was to understand how the transcription factor family *NUCLEAR FACTOR Y* genes integrate the multitude of environmental signals a plant encounters during its lifecycle. Plants, especially angiosperms, have seen a significant expansion in the number of *NF-Y* genes in their genome when compared to animals and fungi. This expansion in gene number has led to the possibility of 1000 unique NF-Y complexes in the model plant species Arabidopsis (Siefers et al., 2009; Laloum et al., 2012). Because of this level of complexity, no complete NF-Y trimer has been described in plants. Here we used reverse genetic approaches to uncover the function of several *NF-Y* genes.

Previous studies identified *NF-YB2* and *NF-YB3* as playing additive roles in the activation of photoperiod dependent flowering in Arabidopsis (Kumimoto et al., 2008). Here we discovered that *NF-YC3*, *NF-YC4* and *NF-YC9* have overlapping function in the control of flowering, marking the first time even two NF-Y subunits were genetically defined for a particular process. In addition, we demonstrated that NF-YC3, NF-YC4 and NF-YC9 could physically interact with NF-YB2 and NF-YB3. Even in paring the possible *NF-Y* genes down to two *NF-YBs* and three *NF-YCs*, this leads to the possibility that at least 6 and as many as 60 different NF-Y complexes could promote flowering (i.e., there are still 10 *NF-YA* left to screen in flowering). Although the reduction from 1000 possible

combination to 60 is significant, to fully test how NF-Y complex formation affects flowering, the identification of the appropriate *NF-YA* genes will be necessary.

One of the major outstanding questions in the plant *NF-Y* field is what are the advantages in having such a diverse set of NF-Y complexes for one response. One explanation for of this may be that different NF-Y complexes have various promoter binding affinities. Testing this hypothesis will greatly enhance our understanding of how NF-Y function in plants and may shed light on structural modifications of NF-Y that affect DNA binding.

The flowering time pathway is an attractive system to start testing some of these hypotheses related to *NF-Y*. *FLOWERING LOCUS T* (*FT*) is one of the few clear direct targets of NF-Y; in addition the minimal functional *FT* promoter region has recently been defined in Arabidopsis (Adrian et al., 2010). Testing the variable DNA binding hypothesis of *NF-Y* is contingent upon defining the promoter element(s) responsible for recruiting *NF-Y.* Directed experiments by mutational analysis of the *FT* promoter will be necessary to define the functional NF-Y binding motifs. The study of how *NF-Y* affect flowering not only extends our knowledge of how plants interact with the environment but will widen our understanding *NF-Y* biochemical function.

In addition to the discovery of the *NF-YC* subunits involved in flowering, we demonstrated that these NF-YCs could physically interact with CONSTANS

(*CO*), a master regulator of flowering. Further we showed that *NF-Y* is necessary for the ability of *CO* to drive flowering. Overexpression of *CO* in wild-type plants leads to precocious flowering and this ability to dive flowering is lost in the *nf-yc triple* mutant backing ground. These data argue that NF-Y form the core of a transcriptional complex at the *FT* promoter to activate flowering in response to inductive photoperiods. How NF-Y activity is regulated to determine flowering time remains an open question.

While the functional interactions between NF-Y and CO are clear, our unpublished data, as well as published results by us and other groups, indicate that NF-Y interact with many members of the CONSTANS-Like (COL) family. The *COL* gene family consists of approximately 40 members, most of which have no known function (Griffiths et al., 2003). Yeast 2-hybrid screens to isolate direct interactors of NF-YC9 discovered *COL3*, which has a known role in light regulated seedling growth. Specifically, mutants for *COL3* are hyposensitive in de-etiolation responses to short day and high fluence rate red light conditions (Datta et al., 2006). We hypothesized that if NF-Y are required for *COL3* function, comparable to *CO*, then *NF-Y* mutants would also show de-etiolation phenotypes under short days and red light. Initial studies have shown that *nf-yc* and *nf-yb* mutants do show similar light regulated growth defects to *col3* mutants. Studies of both *NF-Y* and *COL3* as related to light regulated growth are in their infancy and a more thorough study like the one presented here for the *NF-YC/CO* complex will be necessary to solidify their functional association.

Nevertheless, these data indicate that the *NF-Y/CO* module is likely to be widespread, increasing the already impressive NF-Y complex diversity.

To extend our knowledge of *NF-Y* beyond flowering we performed global transcription profiling experiments on the late flowering *nf-yc3/c4/c9* triple mutant using Affymetrix microarray technology. Subsequent analysis of the microarray data revealed an enrichment of bZIP promoter binding sites in the misregulated gene set. These data complement results obtained from yeast 2-hybrid screens where bZIP proteins were isolated using either NF-YB2 or NF-YC9 as bait. In particular bZIP proteins associated with abscisic acid (ABA) signaling were isolated. Previous findings in plants have shown that NF-Y interact with bZIPs in regulating ABA signaling and endoplasmic reticulum stress responses.

This led to the hypothesis that *NF-YB2*, *NF-YB3*, *NF-YC3*, *NF-YC4* and *NF-YC9* could regulate ABA dependent phenotypes. Mutant lines were tested in a variety of ABA signaling assays. *NF-YC3*, *NF-YC4* and *NF-YC9* appear to play a role in ABA regulated germination responses. Interestingly, unlike in flowering where these three *NF-Ys* have overlapping roles, in response to ABA they show opposing phenotypes. *NF-YC3* and *NF-YC9* inhibit germination while *NF-YC4* activates germination. This data marks the second time *NF-Y* have been shown to have opposing roles in the same process. Over-expression of either *NF-YB2* or *NF-YB3* activate flowering. This is in contrast to over-expression of *NF-YB1*

that represses flowering. Currently there are no mechanistic details for how these highly conserved proteins can drive opposing phenotypes. One hypothesis is that although NF-Ys interact promiscuously within the family, interactions with transacting factors such as bZIP and COL are complex specific. We provide evidence here that supports this hypothesis. NF-YB2 is the lone NF-YB to interact with the bZIP protein ABF3 in yeast 2-hybrid assays. This result was curious because the conserved domains of the tested NF-YB are very similar, showing at least 80% identity across the 97 amino acid motif. This led to the hypothesis that the non-conserved N- and C-terminal domains of the NF-YB must be driving the interaction specificity. We tested this hypothesis using partial clones representing different regions of N-YB2 as well as chimeric clones of NF-YB2 and NF-YB10. This led to the conclusion that while a full length “NF-YB” is required for the ABF3 interaction, the N-terminal region of NF-YB2 is required for the interaction. This presents an attractive hypothesis for expansion and maintenance of the NF-Y families in plants, that different NF-Y drive specific interaction with process-specific bZIP factors. This hypothesis is also applicable to processes where the *COL* gene family is concerned.

The data presented here adds to the expanding roles *Nuclear Factor Y* transcription factors play in plant growth and development. This research not only builds toward answering many of the outstanding questions revolving around *NF-Y*, but also opens important new avenues of study. The progression of research on NF-Y in plant development will rely profoundly on the ability to

discover relevant *in vivo* trimeric complexes and validated direct targets of these transcription factors. Work towards these ends will greatly improve our understanding of not only the working of NF-Y but also how plants can regulate their development to the corresponding environment. With population growth and global climate change putting strain on agricultural production, a deep understanding of plants will be necessary. As *NF-Y* have demonstrated ability to positively affect the both yield and quality of food crops these studies are of utmost relevance.

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