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The *Arabidopsis* Floral Homeotic Proteins APETALA3 and PISTILLATA Negatively Regulate the *BANQUO* Genes Implicated in Light Signaling [W]

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The *Arabidopsis thaliana* MADS box transcription factors APETALA3 (AP3) and PISTILLATA (PI) heterodimerize and are required to specify petal identity, yet many details of how this regulatory process is effected are unclear. We have identified three related genes, *BHLH136*/*BANQUO1* (*BNQ1*), *BHLH134*/*BANQUO2* (*BNQ2*), and *BHLH161*/*BANQUO3* (*BNQ3*), as being directly and negatively regulated by AP3 and PI in petals. *BNQ1*, *BNQ2*, and *BNQ3* encode products belonging to a family of atypical non-DNA binding basic helix-loop-helix (bHLH) proteins that heterodimerize with and negatively regulate bHLH transcription factors. We show that *bnq3* mutants have pale-green sepals and carpels and decreased chlorophyll levels, suggesting that *BNQ3* has a role in regulating light responses. The *ap3 bnq3* double mutant displays pale second-whorl organs, supporting the hypothesis that *BNQ3* is downstream of *AP3*. Consistent with a role in light response, we show that the *BNQ* gene products regulate the function of *HFR1* (for *LONG HYPOCOTYL IN FAR-RED1*), which encodes a bHLH protein that regulates photomorphogenesis through modulating phytochrome and cryptochrome signaling. The *BNQ* genes also are required for appropriate regulation of flowering time. Our results suggest that petal identity is specified in part through downregulation of *BNQ*-dependent photomorphogenic and developmental signaling pathways.

INTRODUCTION

Arabidopsis thaliana petals are simple laminar floral organs; the white petal blades lack chlorophyll and, at maturity, possess characteristic conical epidermal cells on their adaxial surfaces (Irish, 2008). The appropriate specification of petal identity depends on the activities of two MADS box–containing transcription factors, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) (Bowman et al., 1989; Jack et al., 1992; Goto and Meyerowitz, 1994; Krizek and Meyerowitz, 1996). The expression patterns of *AP3* and *PI* depend upon the activity of the meristem identity genes *LEAFY* and *AP1*, which encode transcription factors, in conjunction with the activity of *UNUSUAL FLORAL ORGANS*, encoding an F-box–containing protein (Ng and Yanofsky, 2001; Lamb et al., 2002; Chae et al., 2008). In turn, AP3 and PI form an obligate heterodimer necessary for DNA binding, nuclear localization, and consequent transcriptional regulation of suites of downstream target genes (McGonigle et al., 1996; Riechmann et al., 1996b; Yang et al., 2003b). The AP3/PI heterodimer appears to act together with other MADS box proteins, presumably as components of higher-order protein complexes, to regulate organ-specific differentiation processes (Pelaz et al., 2000; Honma and Goto, 2001). In petals, these processes appear to depend on the combined activities of AP3 and PI in conjunction with the AP1 and SEPALLATA (SEP) MADS box proteins (Pelaz et al., 2000, 2001; Honma and Goto, 2001).

AP3 and *PI* are expressed throughout the petal until late stages of petal differentiation, and continued and ubiquitous expression of these organ identity genes appears to be required throughout the petal for normal development to ensue (Bowman et al., 1989; Goto and Meyerowitz, 1994; Jack et al., 1994; Jenik and Irish, 2001). These observations imply that *AP3* and *PI* act to regulate spatially and temporally distinct subsets of target genes during petal development and differentiation. Although many putative AP3 and PI targets have been identified through microarray and other analyses (Sablowski and Meyerowitz, 1998; Zik and Irish, 2003; Wellmer et al., 2004; Sundstrom et al., 2006; Alves-Ferreira et al., 2007; Peiffer et al., 2008), only a few such target genes have been experimentally verified. These include *AP3* and *PI* themselves, which are autoregulated in a positive feedback loop (Goto and Meyerowitz, 1994; Jack et al., 1994). Regulation of *AP3* is direct, since the AP3/PI heterodimer can bind to CArG box consensus sequences in the *AP3* promoter and *AP3* can be activated by AP3 and PI without de novo protein synthesis (Jack et al., 1992; Goto and Meyerowitz, 1994; Hill et al., 1998; Tilly et al., 1998; Sundstrom et al., 2006). *PI* regulation, however, is likely to be indirect since de novo protein synthesis is required for AP3/PI-dependent regulation of *PI* (Honma and Goto, 2000). *NAP* (for *NAC-LIKE, ACTIVATED BY AP3/PI*), a gene that is involved in the transition between the cell division and cell expansion phases during the growth of petals and stamens and in promoting senescence, has also been shown to be positively regulated by AP3 and PI (Sablowski and Meyerowitz,

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1998; Guo and Gan, 2006). In addition to positive regulation, AP3 and PI have also been shown to act as negative regulators of *AP1*, suggesting that complex feedback regulatory mechanisms are important for appropriate specification of organ identity (Sundstrom et al., 2006). AP3 and PI also negatively regulate the expression of two GATA-type zinc finger genes, *GNC* (for *GATA, NITRATE-INDUCIBLE, CARBON-METABOLISM-INVOLVED*) and *GNC-LIKE* (*GNL*), which in turn regulate a suite of sugar response and nitrate metabolism genes, providing a link between organ development and nutrient sensing (Mara and Irish, 2008).

In this study, we have identified three closely related genes, *BANQUO1* (*BNQ1*), *BNQ2*, and *BNQ3*, that are negatively regulated by AP3 and PI. *BNQ1*, *BNQ2*, and *BNQ3* encode products that are members of the basic helix-loop-helix (bHLH) family of transcriptional regulators. The *Arabidopsis* genome encodes >160 bHLH proteins that have been variously grouped into 15 to 25 subfamilies (Heim et al., 2003; Toledo-Ortiz et al., 2003; Li et al., 2006; Pires and Dolan, 2010). These proteins are characterized by a basic domain of ~15 to 17 amino acids responsible for DNA binding and an HLH region required for dimerization and consisting of two amphipathic α-helices joined by a loop of variable length (Ellenberger et al., 1994; Jones, 2004). However, the *BNQ1*, *BNQ2*, and *BNQ3* gene products have fewer basic amino acids in their basic domains and lack the amino acids (Glu-13/Arg-17) that are critical for DNA binding of canonical bHLH proteins (Toledo-Ortiz et al., 2003). This class of non-basic bHLH proteins, as exemplified by the human Id-1 (Inhibitor of DNA binding-1) protein, is thought to act as dominant-negative regulators of DNA binding bHLH transcription factors (Massari and Murre, 2000; Norton, 2000).

Here, we show, using loss-of-function and gain-of-function approaches, that *BNQ1*, *BNQ2*, and *BNQ3* have a variety of roles in regulating light responses as well as developmental transitions. These roles include the ability to heterodimerize with, and regulate the activity of, the bHLH protein HFR1 (for LONG HYPOCOTYL IN FAR-RED LIGHT1) that is a critical regulator of light signaling and shade avoidance (Fairchild et al., 2000; Soh et al., 2000; Duek and Fankhauser, 2003; Sessa et al., 2005; Zhang et al., 2008; Hornitschek et al., 2009). Together, our data support a model whereby AP3 and PI influence petal morphogenesis in part through the negative regulation of a family of atypical bHLH proteins that in turn modulate the activity of a number of other signaling pathways, providing a mechanistic link between developmental and physiological responses.

RESULTS

The *BNQ* Genes Are Targets of AP3 and PI

To identify genes directly regulated by AP3/PI, we previously conducted a genome-wide screen using the Affymetrix ATH1 GeneChip array to identify genes whose expression was altered in response to steroid-inducible activation of AP3. We used *35S:AP3-GR 35S:PI ap3-3* transgenic plants that constitutively express *PI* as well as constitutively express a steroid-inducible form of *AP3* in an *ap3-3* mutant background. Prior to dexamethasone (dex) induction, these transgenic plants show an *ap3-3* phenotype. After induction, these plants display a rescue of the *ap3-3* mutant phenotype, as well as partial homeotic conversions of sepals to petals and carpels to stamens, reflecting the combined ectopic expression of *AP3* and *PI* (Sablowski and Meyerowitz, 1998). Application of dex to *35S:AP3-GR 35S:PI ap3-3* plants results in transcriptional upregulation of direct targets of AP3/PI within 4 to 6 h of treatment (Sundstrom et al., 2006; Mara and Irish, 2008).

Previously, we used this transgenic line to conduct microarray experiments (Mara and Irish, 2008). One hundred putative AP3/PI targets, genes whose expression profiles changed in a statistically significant manner after 4 h of dex treatment, were identified (Mara and Irish, 2008) and included *BNQ1*. Previously known as *BHLH136*, *BNQ1* encodes one of ~33 predicted non-DNA binding bHLH proteins in the *Arabidopsis* genome; these proteins are thought to inhibit the function of DNA binding bHLH transcription factors through heterodimerization (Fairman et al., 1993; Bailey et al., 2003; Heim et al., 2003; Toledo-Ortiz et al., 2003; Li et al., 2006). The microarray data indicated that *BNQ1* is downregulated 2.1-fold after induction of AP3 activity, suggesting that *BNQ1* is negatively regulated by AP3 and PI. RT-PCR data corroborate the microarray data, indicating that *BNQ1* expression decreases significantly 4 h after dex treatment of *35S:AP3-GR, 35S:PI, ap3-3* transgenic plants and increases in *ap3-3* and *pi-1* mutant flowers compared with the wild type (Figure 1).

BNQ1 encodes a member of a small subfamily of six atypical bHLH proteins (Figures 2A and 2B) that together form a strongly supported subclade within the larger bHLH family (see Supplemental Figure 1 online). Included in this subclade are *BNQ2*, *BNQ3* (previously called *BHLH134* and *BHLH161*, respectively), *At3g28857*, *KIDARI*, and *BHLH135*. This subfamily of bHLH proteins shows considerable conservation of the HLH protein interaction domain but do not possess the stereotypical basic amino acids of DNA binding bHLH proteins (Figure 2C).

We tested if these other members of this bHLH subfamily were also targets of AP3 and PI. We found that *BNQ2* and *BNQ3* expression levels decreased rapidly, within 1 h, after dex treatment of *35S:AP3-GR, 35S:PI, ap3-3* transgenic plants (Figures 1B and 1C). Consistent with this, *BNQ2* and *BNQ3* expression increased in *ap3-3* and *pi-1* mutant plants compared with the wild type (Figures 1A and 1D). *BNQ2* and *BNQ3* were not recovered in our microarray screen due to fact that *BNQ3* was not represented on the array, and *BNQ2* was listed as below the threshold of detection. Thus, we focused our subsequent analyses on *BNQ1*, *BNQ2*, and *BNQ3* that are all negatively regulated by AP3 and PI. Furthermore, the downregulation of the transcription of all three genes occurs rapidly in response to induction of AP3 activity (Figure 1B), suggesting that the AP3/PI heterodimer may be binding directly to the promoters of each of these *bHLH* genes.

BNQ Genes Are Negatively Regulated by AP3 and PI in Petals

Digital gene expression analyses using the *Arabidopsis* eFP browser, a tool for visualizing publicly available microarray data sets (Winter et al., 2007), indicated that *BNQ1* and *BNQ2* are

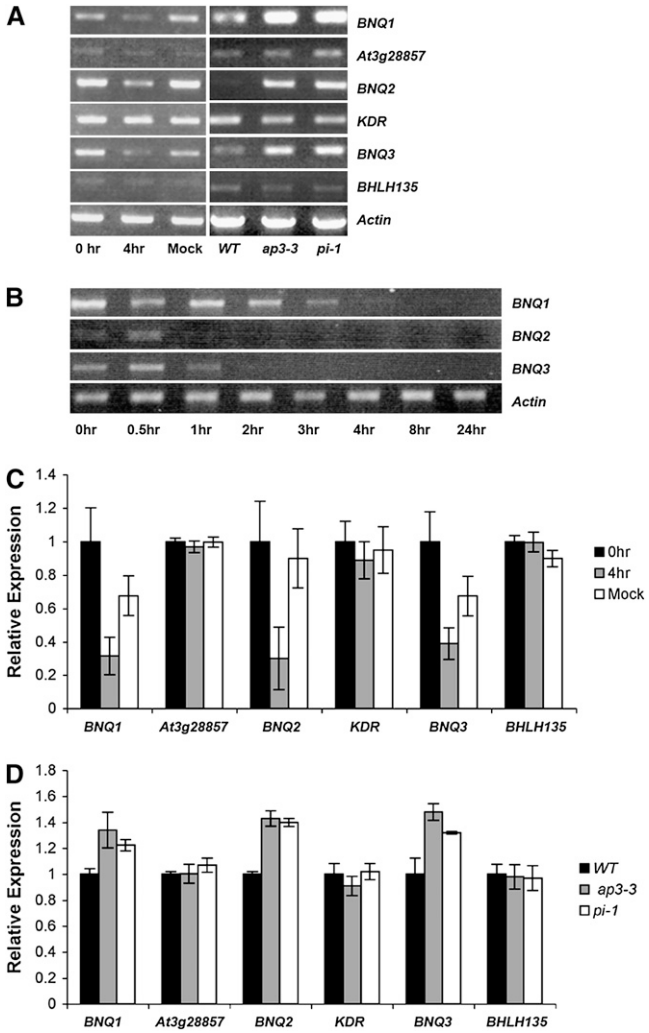

Figure 1. *BNQ1*, *BNQ2*, and *BNQ3* Are Targets of AP3/PI.

(A) Relative expression levels of *BNQ1*, *BNQ2*, and *BNQ3* and other atypical bHLH family members assayed by RT-PCR in 0 and 4 h dex- and mock-treated *35S:AP3-GR 35S:PI ap3-3* flowers and in wild-type (Landsberg *erecta* [L*er*]), *ap3-3*, and *pi-1* mutant flowers. *ACTIN* expression was used as a control.

(B) Time course of relative expression of atypical bHLH genes by RT-PCR in dex-treated *35S:AP3-GR 35S:PI ap3-3* flowers. Dex was applied at time 0 and tissues collected for analysis at times indicated.

(C) Relative expression levels of *BNQ1*, *BNQ2*, and *BNQ3* and family members by RT-PCR in 0 and 4 h dex- and mock-treated flowers. Average expression levels from three biological replicates were normalized to *ACTIN* with 0 h scaled to 1. Standard deviations are shown.

(D) Relative expression levels of *BNQ1*, *BNQ2*, and *BNQ3* and family members by RT-PCR in the linear range in wild-type (L*er*), *ap3-3*, and *pi-1* mutant flowers. Average expression levels from three biological replicates were normalized to *ACTIN* with the wild type scaled to 1. Standard deviations are shown.

expressed at low but detectable levels in most plant tissues and have substantially overlapping expression patterns based on analyses of ATH1 microarray data sets (Schmid et al., 2005) (see Supplemental Figures 2A and 2B online). Similar digital profiling of *BNQ3* expression has been performed using whole-genome tiling arrays (Laubinger et al., 2008) and indicates that *BNQ3* is also expressed in most plant tissues at low but detectable levels (see Supplemental Figure 2C online).

To examine further the mechanisms by which AP3 and PI regulate *BNQ* gene expression, we used in situ hybridizations to characterize the patterns of *BNQ1*, *BNQ2*, and *BNQ3* expression in floral tissues. *BNQ1* expression is detectable in the sepals of wild-type flowers at stage 5 (Figure 3B). Prior to stage 5, *BNQ1* transcripts cannot be detected in the flowers, although expression is strong in cauline leaves (Figures 3A and 3D). Sepal expression continues throughout floral development until stage 12 (Figures 3B to 3F). Weak expression is also detectable in the anthers at later stages (Figure 3F). *BNQ3* expression overlaps considerably with that of *BNQ1*, although *BNQ3* is expressed more broadly in flowers. *BNQ3* is expressed ubiquitously throughout stage 4 floral organ primordia (Figure 3M). From stage 5 onward, *BNQ3* is expressed most strongly in the sepals with some expression detectable in the inner whorls (Figures 3N and 3O). In late stages, *BNQ3* is also strongly expressed in anthers and carpels (Figures 3P to 3R). By contrast, *BNQ2* is expressed weakly throughout the inner whorls of stage 4 wild-type flowers (see Supplemental Figure 3 online). Weak

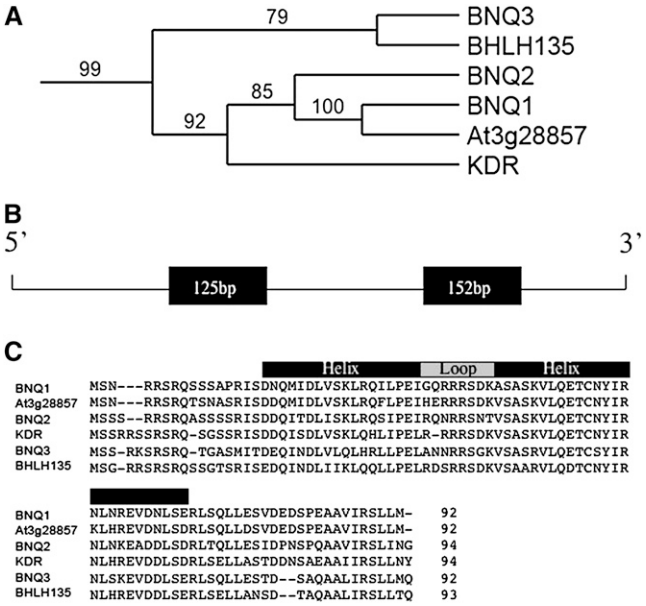

Figure 2. Gene Structure and Amino Acid Sequences of *BNQ* Family Members.

(A) Neighbor-joining analysis of the *BNQ* subclade (see Supplemental Figure 1 online for complete analysis). Bootstrap values of 1000 replicates are shown.

(B) Gene structure of *BNQ1*. Black boxes represent the two exons.

(C) Alignment of amino acid sequences of *BNQ* related family members. The HLH domain is indicated by black and gray boxes.

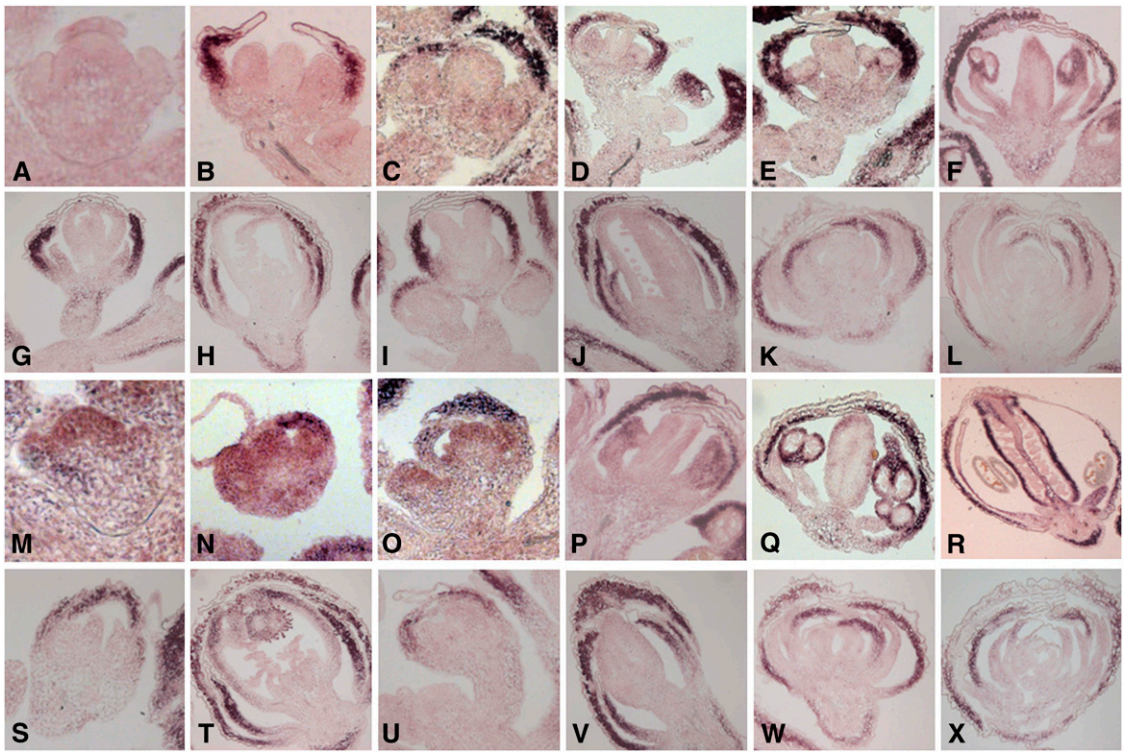

Figure 3. In Situ Expression Analyses of *BNQ* Family Members.

(A) to **(F)** Expression (indicated by purple color) of *BNQ1* in wild-type (L*er*) flowers at stage 4 **(A)**, stage 5 **(B)**, stage 6 **(C)**, stage 7 **(D)**, stage 8 **(E)**, and late-stage **(F)** flowers.
(G) to **(L)** Expression of *BNQ1* in various mutant backgrounds: in approximately stage 8 **(G)** and stage 12 **(H)** *ap3-3* mutant flowers, in approximately stage 6 **(I)** and stage 8 **(J)** *pi-1* mutant flowers, and in approximately stage 8 **(K)** and stage 12 **(L)** *ag-1* mutant flowers.
(M) to **(R)** Expression of *BNQ3* in wild-type flowers at stage 4 **(M)**, stage 5 **(N)**, stage 6 **(O)**, stage 7 **(P)**, stage 8 **(Q)**, and late-stage **(R)** flowers.
(S) to **(X)** Expression of *BNQ3* in various mutant backgrounds: in approximately stage 6 **(S)** and stage 12 **(T)** *ap3-3* mutant flowers, in approximately stage 6 **(U)** and stage 12 **(V)** *pi-1* mutant flowers, and in approximately stage 8 **(W)** and stage 12 **(X)** *ag-1* mutant flowers.

expression of *BNQ2* persists in the stamen and carpel primordia until stage 8, but by stage 9, expression is no longer detectable (see Supplemental Figure 3 online).

To test whether AP3 and PI restrict the spatial domains of *BNQ1* and *BNQ3* expression, we examined their expression patterns in *ap3-3* and *pi-1* mutant flowers. In stages 6 to 8 of *ap3-3* and *pi-1* flowers, *BNQ1* expression is observed in the first whorl of sepals (Figures 3G and 3I). In stage 12 *ap3-3* and *pi-1* flowers, *BNQ1* expression is also found in the second-whorl organs (Figures 3H and 3J). To determine if *BNQ1* expression is position dependent or tissue specific, we monitored its expression in *ag-1* mutant flowers in which stamens are transformed into petals and the fourth whorl differentiates into a new flower consisting only of sepals and petals (Bowman et al., 1989). We found that *BNQ1* is expressed in each whorl of sepals regardless of position (Figures 3K and 3L). Similarly, in *ap3-3* and *pi-1* mutant flowers, *BNQ3* is expressed in the first whorl, and by stage 12, its expression domain expands into the second whorl of sepals, indicating that AP3 and PI repress *BNQ3* in the second whorl (Figures 3S to 3V). This repression is tissue specific and not whorl specific, since in *ag-1* mutant flowers, *BNQ3* is expressed both in first-whorl sepals and in ectopic fourth-whorl sepals (Figures 3W and 3X). Thus, these data indicate that AP3 and PI repress the expression of both *BNQ1* and *BNQ3* in developing petals.

To determine if the AP3/PI heterodimer binds to the promoters of the *BNQ* genes, we performed chromatin immunoprecipitation (ChIP) assays. The AP3/PI heterodimer has been shown to bind to a 10-bp conserved DNA region called the CArG box [CC(A/T)$_6$GG] (Schwarz-Sommer et al., 1992; Riechmann et al., 1996a; Hill et al., 1998; Tilly et al., 1998). Allowing for a 1-bp mismatch, we identified a number of CArG-like boxes present in the promoter regions of *BNQ1*, *BNQ2*, and *BNQ3* and tested if PI can bind to these sequences (Figure 4). We extracted nuclei from wild-type and *35S:PI-HA* epitope-tagged transgenic plants and immunoprecipitated with either α-HA antibody or normal mouse serum. Immunoprecipitated DNA from three independent biological replicates was used in ChIP-PCR reactions with primers designed around each CArG-like box to monitor enrichment (Figure 4A). As a positive control, we confirmed binding of PI to CArG3, an autoregulatory region in the *AP3* promoter (Hill et al., 1998). No enrichment was detected in the negative controls, *PI* (an indirect target of AP3/PI; Honma and Goto, 2000) or *AST101* (a root-specific gene; Takahashi et al., 2000) (Figures 4B and 4C). We could detect an enrichment of a 250-bp fragment in the

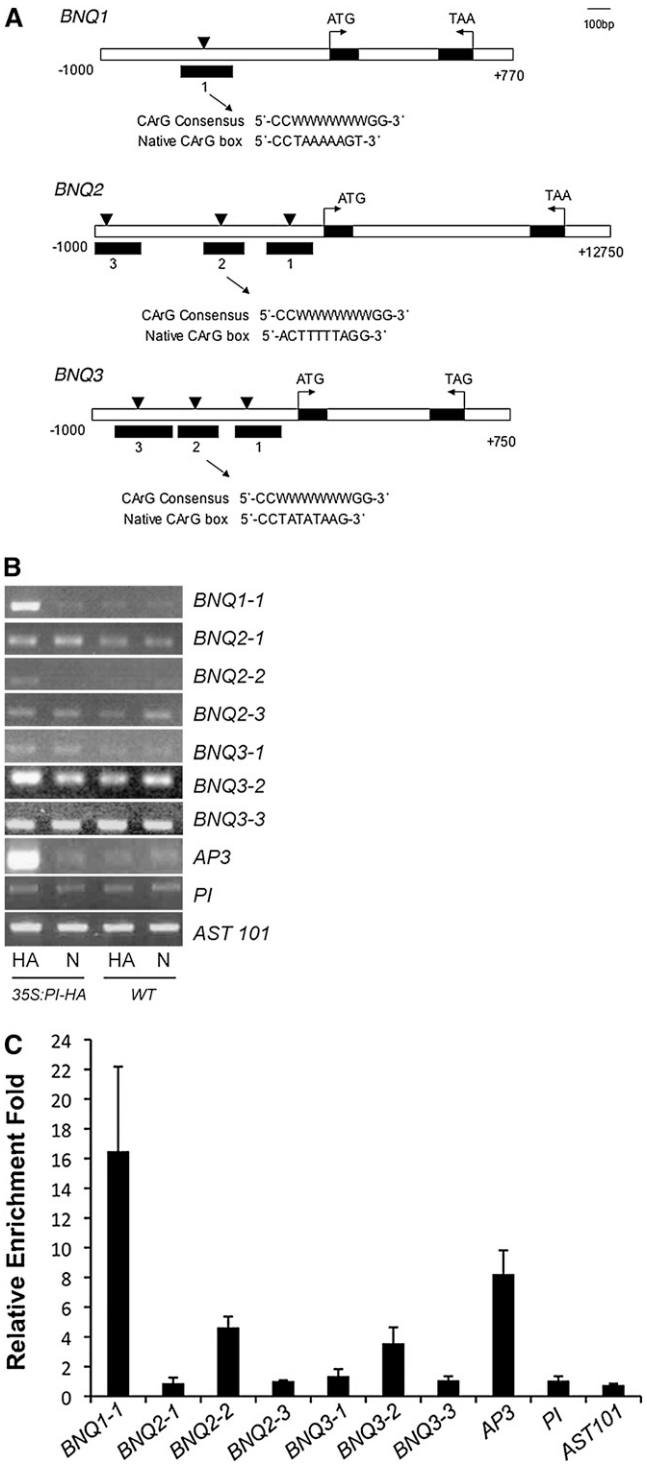

Figure 4. AP3/PI Proteins Are Associated with Promoter Elements of the *BNQ1*, *BNQ2*, and *BNQ3* Genes.

(A) CArG boxes in the promoters of *BNQ1*, *BNQ2*, and *BNQ3*. Black triangles indicate the position of the CArG box, and the black boxes indicate the regions amplified by the primers used for ChIP-PCR. Sequences shown below each schematic indicate the sequence present in the specific CArG box indicated.

(B) ChIP-PCR for each of the regions indicated in **(A)**, as well as for CArG 2 in the *AP3* promoter and control promoter regions of the *PI* and *AST101* genes. Nuclear extracts from 35S:PI-HA and wild-type (L*er*) plants were immunoprecipitated with anti-HA antibody (HA) or normal serum (N).

(C) Relative enrichment levels in the *BNQ1*, *BNQ2*, *BNQ3*, *AP3*, *PI*, and *AST101* promoters. Black bars indicate the enrichment fold change based on three replicates that were normalized to wild-type samples immunoprecipitated with normal serum scaled to 1. Standard deviations are shown.

BNQ1 promoter region that contains the CArG-like box in *35S:PI-HA* extracts precipitated with α-HA antibodies compared with controls (Figure 4C). We also detected an enrichment of a 169-bp region spanning CArG box 2 present in the *BNQ2* promoter and a slight enrichment of a 216-bp region containing CArG box 2 present in the *BNQ3* promoter (Figure 4C). However, we could not detect any enrichment of any of the other CArG-like boxes present in the promoters of *BNQ2* and *BNQ3* (Figure 4C). Thus, *BNQ1*, *BNQ2*, and *BNQ3* appear to be direct targets of PI, presumably through binding of the AP3/PI heterodimer to a CArG box sequence in the promoters of each of these genes.

Roles of *BNQ* Genes in Chlorophyll Accumulation and Floral Induction

A T-DNA insertional mutation in the second predicted helix of the *BNQ3* coding region was obtained from the SALK collection (Alonso et al., 2003) and backcrossed four times to remove exogenous lesions. Homozygous *bnq3* (SALK 098881) mutants have undetectable levels of transcripts, suggesting that the mutation is a complete loss-of-function allele (Figure 7A).

The sepals and carpels of homozygous *bnq3* mutant plants are pale yellow or white, while the inflorescence stems and siliques are purple (Figure 5). The floral organs appear to be morphologically normal but are somewhat smaller than wild-type organs (Figures 5C to 5F). To test the genetic relationship of *AP3* and *BNQ3*, we generated *ap3-3 bnq3* double mutant plants. While *ap3-3* single mutant plants display green sepaloid organs, the *ap3-3 bnq3* double mutant flowers have pale yellowish second-whorl organs, consistent with negative regulation of *BNQ3* by *AP3* (Figure 6).

Consistent with the pale phenotype of *bnq3* mutants, we found that flowers from such plants had decreased levels of chlorophyll compared with wild-type flowers (Figure 7B). We also detected lower amounts of chlorophyll in the cauline leaves, stems, and siliques of the *bnq3* mutant plants (Figure 7B). Complementation tests in which homozygous *bnq3* plants were transformed with a *35S:BNQ3* construct produced normal flowers with green sepals (see Supplemental Figure 4 online), demonstrating that the T-DNA insertion in *BNQ3* is responsible for the pale phenotype.

We used RNA interference (RNAi) to knock down *BNQ1* and *BNQ2* expression. We generated 23 *BNQ1* RNAi lines to see if we could detect a loss-of-function phenotype; there were no obvious phenotypes in any of these lines (Figure 7A; data not shown).

Twenty-one *BNQ2* RNAi transgenic lines were generated, and none of the *BNQ2* RNAi lines showed an obvious mutant phenotype (Figure 7A; data not shown). We assayed the relative expression of *BNQ1* (or *2*) in the corresponding RNAi lines

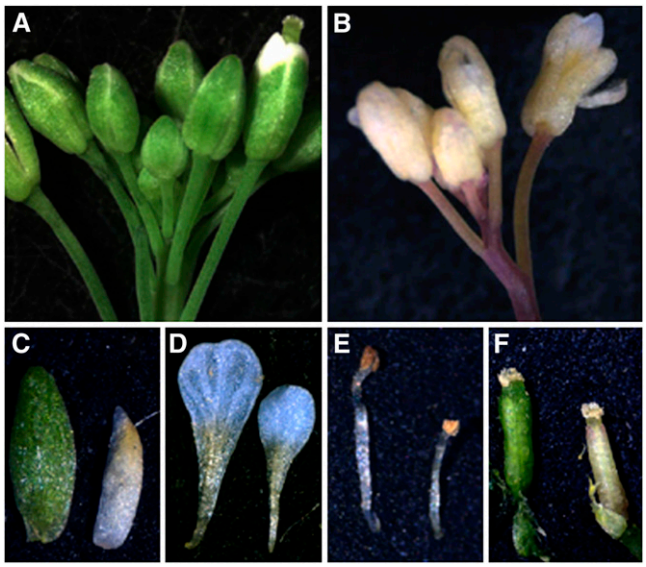

Figure 5. Mutational Analysis of *BNQ3.*

(A) Wild-type (Columbia [Col]) flower buds.
(B) *bnq3* mutant flower buds.
(C) Sepals from wild-type (left) and *bnq3* mutant (right) flowers.
(D) Petals from wild-type (left) and *bnq3* mutant (right) flowers.
(E) Stamens from wild-type (left) and *bnq3* mutant (right) flowers.
(F) Carpels from wild-type (left) and *bnq3* mutant (right) flowers.

compared with the expression in the wild type normalized to actin and used lines with a significant reduction in *BNQ* gene expression for further experiments (22% of wild-type levels for *BNQ1i* and 19% of wild-type levels for *BNQ2i*). To determine if the *BNQ* genes act redundantly, we generated triple *BNQ1i BNQ2i bnq3* knockdown combinations. However, the triple mutants showed no enhancement of the *bnq3* chlorophyll-deficient mutant phenotype or any other obvious morphological phenotypes (Figure 7B). These results suggest that *BNQ3* has a nonredundant role in chlorophyll deposition and that functions of the other *BNQ* family members may be obscured by redundancy with other, as yet uncharacterized, atypical bHLH proteins.

We also observed that *bnq3* mutant plants flower later than the wild type (Figure 7C). On the other hand, transgenic plants containing the *35S:BNQ3* construct resulted in bolting and flowering considerably earlier than the wild type (Figures 8B and 8D). Similarly, constitutive expression of either *BNQ1* or *BNQ2* under control of the *35S* promoter also resulted in early flowering (Figures 8B and 8D). Despite early flowering, these *BNQ1*, *BNQ2*, and *BNQ3* overexpression lines showed no obvious defects in floral morphology. Thus, in addition to *BNQ3*, *BNQ1*, and *BNQ2* may also normally function to promote the transition to flowering.

BNQ Genes Act as Regulators of Light Signaling

The *35S:BNQ1*, *35S:BNQ2*, and *35S:BNQ3* lines also displayed phenotypes that suggested that these *bHLH* genes have a role in light signal transduction. Transgenic seedlings constitutively expressing each of the *BNQ* genes had elongated hypocotyls compared with the wild type when grown under white light (Figures 8A to 8C). These phenotypes were predominantly red light dependent (Figure 8C), indicating that this response is likely to be mediated to a large extent by phytochromes A and B (Smith, 2000; Tepperman et al., 2004).

In *Arabidopsis* seedlings, both the cryptochromes, which absorb blue/UV-A light, and the phytochromes, which sense red/far-red light, are necessary for normal hypocotyl development (Briggs and Olney, 2001; Wang and Deng, 2004). *HFR1* encodes a bHLH protein that is required for both phytochrome- and cryptochrome-dependent light signal transduction in seedlings and during shade avoidance responses (Fairchild et al., 2000; Duek and Fankhauser, 2003; Sessa et al., 2005; Hornitschek et al., 2009). To investigate the potential relationship between the *BNQ* and *HFR1* gene products, we performed yeast two-hybrid analyses. We found that BNQ1, BNQ2, and BNQ3 each were capable of physically interacting with HFR1 (Table 1). In addition to HFR1, several other bHLH proteins have been implicated in light signal transduction in *Arabidopsis*, including PHYTOCHROME INTERACTING FACTOR 3-LIKE1 (PIL1), PIL5, PHYTOCHROME INTERACTING FACTOR3 (PIF3), and PIF4 (Huq and Quail, 2002; Kim et al., 2003; Toledo-Ortiz et al., 2003; Huq et al., 2004; Oh et al., 2004; Castillon et al., 2007). We also examined whether BNQ1, BNQ2, and BNQ3 could interact in yeast two-hybrid assays with PIL1, PIL5, PIF3, or PIF4 (Table 1). No such interactions could be detected, suggesting that BNQ1, BNQ2, and BNQ3 specifically interact with the bHLH protein HFR1 to modulate light signaling.

To test further this possibility, we examined whether overexpression of the BNQ proteins could repress HFR1 function in vivo. We constructed *35S:BNQ; 35S:HFR1* doubly transgenic plants (i.e., *35S:BNQ1 35S:HFR1*, *35S: BNQ2 35S:HFR1*, and *35S:BNQ3 35S:HFR1*) and measured the resulting hypocotyl lengths. The *35S:HFR1* plants have slightly shorter hypocotyls compared with the wild type, while *35S:BNQ1*, *35S:BNQ2*, or *35S:BNQ3* plants have elongated hypocotyls (Figures 9A and 9B). We found that the doubly transgenic plants had hypocotyls that were similar in length to *35S:BNQ1*, *35S:BNQ2*, or *35S: BNQ3* plants and longer than *35S:HFR1* or wild-type plants (Figures 9A and B). This suppression of the *35S:HFR1* hypocotyl phenotype indicates that the BNQ proteins can interact with HFR1 in vivo, presumably by heterodimerizing with, and repressing, HFR1 activity. Furthermore, these data suggest that the *BNQ* gene products interact with other, presumably as yet uncharacterized, bHLH proteins to regulate hypocotyl growth since the *35S:BNQ 35S:HFR* seedlings display longer hypocotyls than do *hfr1* mutants.

DISCUSSION

AP3 and PI Negatively Regulate the Expression of a Family of *bHLH* Genes

In this study, we identified *BNQ1*, *BNQ2*, and *BNQ3* as genes that are negatively regulated by AP3 and PI. We demonstrated that, in the absence of *AP3* or *PI* activity, *BNQ1* and *BNQ3* become ectopically expressed in the second whorl. These

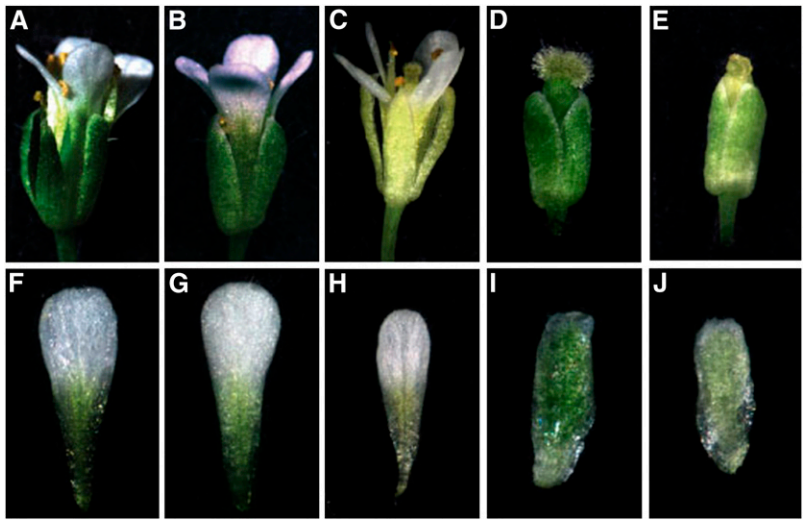

Figure 6. Floral Phenotypes of *ap3-3 bnq3* Double Mutant.

(A) to **(E)** Individual flowers of L*er* **(A)** and Col **(B)** with green sepals; by contrast, *bnq3* **(C)** displays pale-yellow sepals. The *ap3-3* mutant **(D)** has green first and second whorl organs compared with the *ap3-3 bnq3* double mutant **(E)**, which is pale.
(F) to **(J)** The second-whorl organs of *Ler* **(F)** and Col **(G)** have morphologically normal petals. The *bnq3* **(H)** petals also appear normally shaped. The second-whorl organs of *ap3-3* **(I)** are sepaloid and green, while the *ap3-3 bnq3* **(J)** second-whorl organ is pale.

observations suggest that the AP3/PI heterodimeric transcription factor may have important roles in repressing a family of atypical bHLH proteins to ensure the proper development of petals in the second whorl. Furthermore, this repression appears to be direct, based on both the rapid repression of expression of all three *BNQ* genes upon the activation of *AP3* function, as well as through ChIP assays that demonstrate that PI can directly associate with CArG boxes present in the *BNQ1*, *BNQ2*, and *BNQ3* promoters.

Although AP3 and PI act to specify both petal and stamen identity, the AP3- and PI-dependent negative regulation of the *BNQ* genes appears to be petal specific, in that *BNQ1* and *BNQ3* expression can be observed in late stage stamens. Presumably, this is due to the fact that the AP3/PI heterodimer can form higher-order transcriptional complexes with the AP1 and SEP MADS box proteins to direct petal development specifically (Pelaz et al., 2000, 2001; Honma and Goto, 2001). Furthermore, negative regulation by the AP3/PI heterodimer may be achieved via formation of transcription complexes containing corepressors or through affecting histone modifications of target gene promoter regions. Although potential AP3/PI corepressors have not yet been identified, *SEUSS* and *LEUNIG* encode components of a corepressor complex that acts in conjunction with other floral organ identity MADS box gene products to regulate petal development (Franks et al., 2006; Sridhar et al., 2006). Furthermore, the recruitment of a histone deacetylase complex is necessary for the MADS domain protein, AGL15, to act as a transcriptional repressor in vivo (Hill et al., 2008). Since relatively few petal-specific genes have been identified despite multiple microarray analyses (Zik and Irish, 2003; Wellmer et al., 2004), repression as opposed to activation of specific genes by the AP3/PI heterodimer may be a predominant means of petal specification.

Our data suggest that AP3 and PI spatially repress *BNQ1*, *BNQ2*, and *BNQ3* expression in whorl 2 to promote the correct specification of petals. Furthermore, *bnq3* mutants have decreased chlorophyll levels associated with a pale-white phenotype, indicating a requirement for *BNQ3* to promote chlorophyll accumulation. Thus, it appears that AP3 and PI may function in part to abrogate chlorophyll accumulation in the petals to ensure the proper differentiation of these organs. Nonetheless, it is clear that expression of *BNQ3* is not sufficient for chlorophyll accumulation since ectopic expression of *BNQ3* does not result in greening of the petals. Furthermore, ectopic expression of all three *BNQ* genes does not result in petal greening. These results imply that in *ap3* or *pi* mutants, other factors in addition to the *BNQ* gene products are necessary to promote chlorophyll accumulation in the second whorl. Such factors could potentially correspond to the products of other genes that have been shown to be negatively regulated by AP3 and PI, such as *GNC* or *GNL*, which have also been shown to be required for chlorophyll biosynthesis (Bi et al., 2005; Mara and Irish, 2008).

Based on the loss of chlorophyll autofluorescence, redifferentiation of green chloroplasts to colorless leucoplasts in developing petals occurs around stage 12 of *Arabidopsis* flower development (Pyke and Page, 1998). Our observations that *BNQ* gene expression expands into the second whorl only at later stages of flower development in *ap3-3* and *pi-1* flowers is consistent with this observation and suggest that AP3 and PI have specific regulatory roles at later stages of petal organogenesis. The NAC family transcription factor *NAP*, a previously identified direct target of AP3 and PI, has also been proposed to act at later stages of petal differentiation (Sablowski and Meyerowitz, 1998). Similarly, the floral organ identity gene *AGAMOUS* has been shown to regulate directly *SPOROCYTELESS*, encoding a putative transcription factor required for

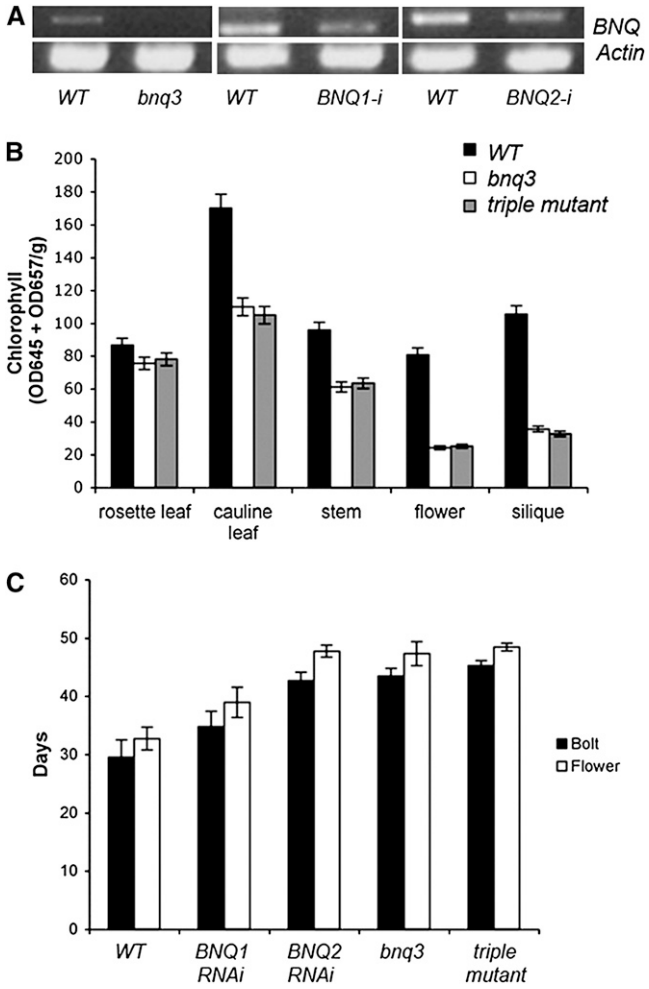

Figure 7. Analysis of *BNQ* Loss-of-Function Phenotypes.

(A) Relative expression of *BNQ1*, *BNQ2*, and *BNQ3* in wild-type (Col) flowers and from corresponding RNAi or mutant flowers. *ACTIN* levels are shown in comparison.

(B) Chlorophyll levels in different tissues from wild-type (Col), *bnq3*, and *bnq* triple mutant (*bnq3 BNQ1-RNAi BNQ2-RNAi*) plants. Standard deviations using three replicates are shown.

(C) Days to bolting and to appearance of first flower are shown for wild-type (Col), *bnq3*, *BNQ1-RNAi*, *BNQ2-RNAi*, and triple mutant plants. Standard deviations from 25 plants scored for each genotype are shown.

microsporogenesis during late stages of flower differentiation (Ito et al., 2004). Together, these observations underscore the idea that the floral organ identity genes regulate different aspects of organogenesis throughout development by regulating the expression of subsidiary transcription factors required for specific differentiation processes.

The *BNQ* Genes Regulate a Variety of Physiological and Developmental Responses

The *BNQ* genes that we have identified belong to an atypical class of bHLH proteins that lack the basic DNA binding domain and the critical amino acids for DNA binding (Toledo-Ortiz et al., 2003). As a consequence, such proteins can form inactive heterodimers with other bHLH proteins, thus modulating activity of their binding partners (Norton, 2000).

At least one BNQ interacting partner appears to be the product of *HFR1*, which itself encodes an atypical bHLH protein (Fairchild et al., 2000). *HFR1* has been shown to be required for

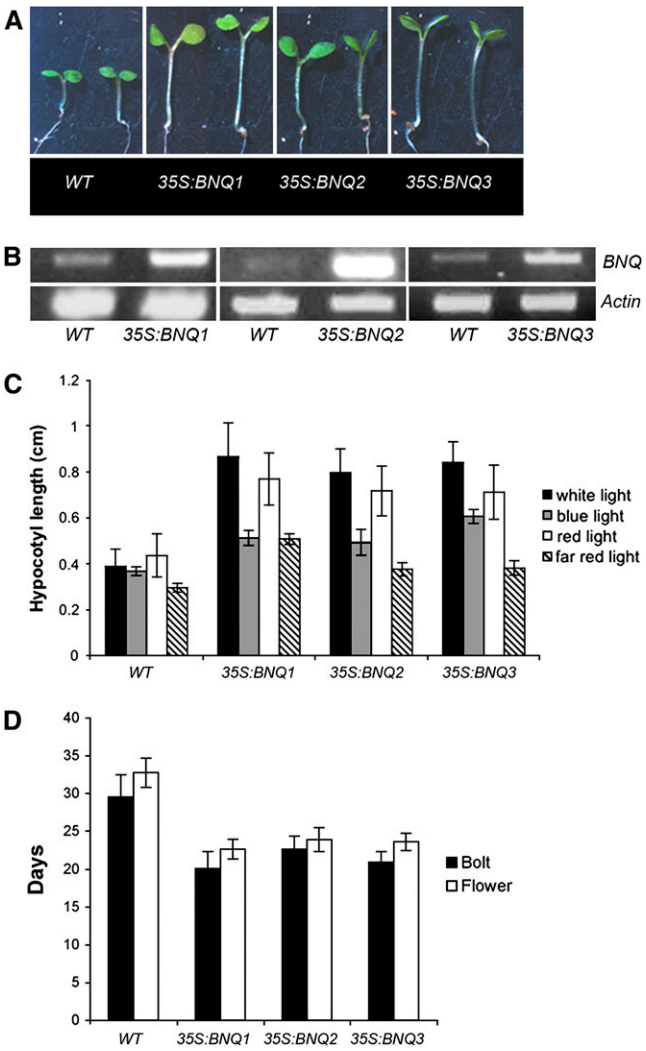

Figure 8. Overexpression Analyses of *BNQ* Genes.

(A) Representative *35S:BNQ1*, *35S:BNQ2*, and *35S:BNQ3* seedlings have elongated hypocotyls compared with the wild type (Col) (two plants each).

(B) *BNQ1*, *BNQ2*, and *BNQ3* expression is increased in the corresponding overexpression lines; *ACTIN* amplification is shown as a control.

(C) Quantified hypocotyl lengths of *35S:BNQ1*, *35S:BNQ2*, and *35S:BNQ3* seedlings under white, blue, red, and far-red light conditions. Standard deviations are shown for 50 plants scored of each genotype.

(D) *35S:BNQ1*, *35S:BNQ2*, and *35S:BNQ3* plants flower earlier than the wild type; days to forming a 1-cm bolt and to first flower opening are shown. Standard deviations are shown for 50 plants scored of each genotype.

Table 1. Yeast Two-Hybrid Analyses of bHLH Gene Products

	HFR1-BD/HFR1-AD	PIL1-BD/PIL1-AD	PIL5-BD/PIL5-AD	PIF3-BD/PIF3-AD	PIF4-BD/PIF4-AD	PGBT9/PGAD424
BNQ1-AD/BNQ1-BD	+/+	−/−	−/−	+/−	+/−	−/−
BNQ2-AD/BNQ2-BD	+/−	−/−	−/−	+/−	+/−	−/−
BNQ3-AD/BNQ3-BD	+/−	−/−	−/−	+/−	+/−	−/−
PGAD424/PGBT9	−/−	−/−	−/−	+/−	+/−	

Results for combinations of constructs containing either the GAL4 activation domain (AD) or GAL4 binding domain (BD) or vector controls containing the GAL4 activation domain (PGAD424) or GAL4DNA binding domain (PGBT9). + Indicates positive interaction based on β-galactosidase expression; − indicates no detectable interaction.

seedling morphogenesis as well as modulating the vegetative shade avoidance response, through mediating phyA-dependent far-red and cry1-dependent blue light signaling pathways (Fairchild et al., 2000; Soh et al., 2000; Duek and Fankhauser, 2003; Sessa et al., 2005; Jang et al., 2007; Zhang et al., 2008; Hornitschek et al., 2009). *HFR1* transcription itself is regulated by light, in that low R/FR light rapidly induces the expression of *HFR1* in vegetative tissues, that in turn acts as a brake to repress shade avoidance responses through forming non-DNA binding heterodimers with the bHLH proteins PIF4 and PIF5 (Sessa et al., 2005; Hornitschek et al., 2009). HFR1 activity itself is also regulated posttranscriptionally. In seedlings, HFR1 protein levels are affected by light-dependent phosphorylation as well as by COP1 and SPA1 E3 ligase-dependent ubiquitination and subsequent degradation by the 26S proteasome pathway (Duek et al., 2004; Jang et al., 2005; Yang et al., 2005a, 2005b; Park et al., 2008).

We have found that overexpression of the *BNQ* genes results in a long hypocotyl phenotype under red light conditions, although a mild long hypocotyl phenotype in comparison to the wild type was also observed under all light conditions tested (Figure 8). In addition, we have shown that the *BNQ* gene products dimerize specifically with HFR1, and their overexpression can suppress overexpression of HFR1. These observations suggest one mechanism for *BNQ* gene function is to sequester HFR1 and thus promote PIF4- and PIF5-dependent light-regulated processes. In petals, AP3- and PI-dependent downregulation of *BNQ* gene expression would in turn allow for the function of the HFR1 protein; HFR1 activity would then result in downregulation of PIF4- and PIF5-dependent light responses. These observations are also consistent with the approximately 10-fold higher levels of *HFR1* transcripts observed in petals compared with other floral organs (Schmid et al., 2005; Winter et al., 2007). Alternatively, or in addition, the *BNQ* gene products may be modulating the activity of other, as yet uncharacterized, bHLH proteins during petal differentiation.

We have also shown that loss or gain of *BNQ* gene function affects flowering time. This may potentially occur through BNQ-mediated regulation of PIF4 function. PIF4 has been recently shown to be required for normal flowering time, in that *pif4* mutants display an early flowering phenotype under high temperature conditions (Koini et al., 2009). This role of PIF4 is likely to be independent of HFR1 since alteration in HFR1 activity via overexpression of a dominant-negative form of HFR1 does not affect flowering time (Yang et al., 2003a), although the effects of *HFR1* overexpression were not assayed under high temperature conditions. Furthermore, our data indicate that the BNQ gene products do not heterodimerize with PIF4, suggesting that the BNQ gene products may impinge on PIF4 regulation indirectly through other, as yet uncharacterized, bHLH proteins. Also, since the *pif4* early flowering phenotype is apparent only under high temperature conditions, and the *bnq1* late flowering phenotype is apparent at normal (22°C) growth temperatures, it appears that BNQ1 can act through other, PIF4-independent, flowering time regulators as well. Overexpression of *BNQ1* has been implicated in modulating gibberellin signaling (Lee et al., 2006); since the gibberellin signaling pathway is required for flowering (Schwechheimer and Willige, 2009), it may be that the flowering time defects we observed reflect in part alterations in the transcriptional control of gibberellin-dependent responses.

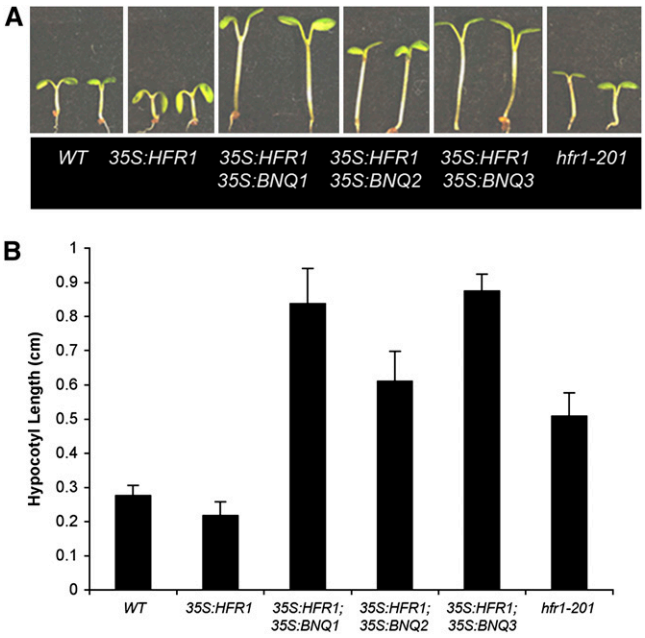

Figure 9. Overexpression of *BNQ1*, *BNQ2*, and *BNQ3* Can Repress the Overexpression Phenotype of *HFR1* in Planta.

(A) Doubly heterozygous *35S:BNQ 35S:HFR1* combinations compared with wild-type, *35S:HFR1*, and *hfr1-201* seedlings.

(B) Quantified hypocotyl lengths of *35S:BNQ 35S:HFR1* combinations compared with wild-type, *35S:HFR1*, and *hfr1-201* seedlings. Standard deviations are shown for 50 plants scored of each genotype.

Overexpression of the *BNQ* genes also has been shown recently to abrogate the vegetative phenotype of the brassinosteroid receptor mutant *bri1-301*, suggesting that the *BNQ* gene products also participate in regulating brassinosteroid signaling through modulating the activity of regulatory bHLH proteins (Wang et al., 2009).

Together, these results imply that the *BNQ* genes are likely acting to modulate multiple exogenous and endogenous cues that are necessary for different aspects of plant development. Given that only a handful of the >160 bHLH genes in *Arabidopsis* have been functionally characterized, our analyses of the *BNQ* genes have uncovered a new role for this subfamily of atypical bHLH proteins. The *BNQ* genes have both overlapping and independent functions in different parts of the plant. These functions include regulation of light signal transduction, chlorophyll accumulation, and the regulation of the floral transition. We suggest that the *BNQ* gene products can regulate these processes in part through modulating the activity of the HFR1 atypical bHLH protein. This regulatory mechanism of modulating the activity of non-DNA binding HLH protein activity through sequestering such proteins through heterodimerization with other non-DNA binding HLH proteins may represent a general strategy to titrate the regulatory roles of such proteins. The downregulation of *BNQ* gene expression specifically in petals through the action of AP3 and PI alters this homeostasis, promoting petal differentiation. Thus, a cascade of transcriptional and postranscriptional negative regulation appears to be one mechanism by which petal morphogenesis is achieved.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana plants were grown on 12:3:1 mix of vermiculite:soil:sand at 22°C under 16-h-light/8-h-dark conditions. The mutant lines (*ap3*-3, *pi-1*, and *ag-1*) and transgenic lines (*ap3-3 35S:PI 35S:AP3-GR*, *35S:AP3* and *35S:PI-HA*) are in the L*er* background. Mutant lines were obtained from the ABRC (Ohio State University). The *ap3-3 35S:PI 35S:AP3-GR* line was a gift from Robert W. M. Sablowski (John Innes Centre, Norwich, UK) (Sablowski and Meyerowitz, 1998). The *35S: PI-HA* line was a gift from Naomi Nakayama (Yale University, New Haven, CT) (Sundstrom et al., 2006).

For dex induction, plants were treated with dex (0.015% silwet, 0.1% ethanol, and 5 μM dex) or mock (0.015% silwet and 0.1% ethanol), collected at various timepoints, and snap frozen in liquid nitrogen. Total RNA was extracted using Trizol (GibcoBRL) according to the manufacturer's instructions, purified using the Qiagen Rneasy kit, and used in subsequent analyses.

The *BNQ3* insertional mutation was identified as a SALK T-DNA insertion line (SALK 098881) and is in the Col background; the mutant was backcrossed four times to remove exogenous mutations. To generate the *ap3-3 bnq3* double mutant, *ap3-3* was crossed with *bnq3* homozygous plants. Following self-pollination of the F1 plants, phenotypic characterization of the double mutants was performed in three F2 families, with 10 *ap3-3 bnq3* double mutants identified out of 144 plants. Representative flowers were dissected and photographed.

For light treatments, seedlings were placed in a standard continuous-white-light growth chamber at 22°C. After 12 h of incubation, seedlings were transferred to various light conditions in growth chambers (Percival Scientific) with fluence rates of 111.0 μmol m^{-2} s^{-1} for far-red light, 150.6 μmol m^{-2} s^{-1} for white light, 172.6 μmol m^{-2} s^{-1} for red light, and 8.1 μmol m^{-2} s^{-1} for blue light.

ChIP and Expression Analyses

For ChIP assays, nuclear extracts were prepared using MC, M1, M2, and M3 buffers as described by Ito et al. (1997) and immunoprecipitations performed as by Mara and Irish (2008). Fractions corresponding to bound and unbound DNA samples were used as templates for ChIP-PCR using primers flanking the CArG-like boxes identified in the promoter regions of each gene using the RSA tools software (rsat.ulb.ac.be/rsat/) (Thomas-Chollier et al., 2008). For ChIP and expression analyses, DNA band intensities from ethidium bromide–stained gels were measured with Molecular Imaging Software 4.0 (Eastman KODAK Company). This software used a Gaussian Curve method with background subtraction to normalize the DNA band intensity, which significantly increases the accuracy of measuring extremely strong or weak DNA bands. This software also directly converts band intensity into DNA content (μg) in a specific DNA band by comparing it to a standard (DNA size marker), which also ensures that bands were measured in the linear range for DNA quantification in a gel image. For RT-PCR, cycle numbers were varied between 20 and 35, and the linear phase of amplification was determined empirically for each reaction by assessing band intensities for different cycle numbers. Gene-specific primers used to analyze expression are listed in Supplemental Table 1 online. The primers used for ChIP-PCR are listed in Supplemental Table 2 online.

In Situ Hybridization

In situ probes were generated by PCR amplification of cDNA using gene-specific primers containing T7 RNA polymerase binding sites. Procedures for probe preparation, sectioning, in situ prehybridization, hybridization, and detection were performed as described previously (Zondlo and Irish, 1999; Mara and Irish, 2008). The primers used for in situ probes are listed in Supplemental Table 3 online.

Transgenic and SALK Line Analyses

All RNAi lines were generated in the Col background using the Gateway system (Invitrogen) vectors pK7GWIWG2 (II) and pH7GWIWG2 (II) and protocol. Gene-specific primers used to amplify ~300-bp coding regions of *BNQ1* and *BNQ2* to insert into the vectors are listed in Supplemental Table 4 online. Reduction in expression of RNAi transgenic lines was assessed by comparing the relative expression of *BNQ1* (or *2*) in the corresponding RNAi line to the expression in the wild type (scaled to 1) and normalized to actin, using three replicates. To construct the plasmids used in genetic complementation and overexpression analysis, the full-length cDNA of each *BNQ* gene was amplified with the corresponding primers listed in Supplemental Table 5 online. The PCR fragment was digested with *Bam*HI and *Xba*I and inserted into the binary vector p235 (Jenik and Irish, 2001), a derivative of pPZP221 (Hajdukiewicz et al., 1994) containing the 35S promoter from the cauliflower mosaic virus. These constructs were introduced into *Agrobacterium tumefaciens* and transformed into *Arabidopsis* plants by floral dip. For genetic complementation, both p235 and *35S:BNQ3* were transformed into *bnq3* mutants, whereas L*er* plants were used for the overexpression analysis of *BNQ* genes. Transgenic plants were selected on half-strength Murashige and Skoog plates containing gentamicin.

Homozygous SALK lines were identified by PCR genotyping for the presence of the T-DNA insertion. RNA was extracted from homozygous plants using Trizol (GibcoBRL) according to the manufacturer's instructions. RT-PCR analysis, as described above, was used to check for abolishment of the transcript. The primers used to verify the *BNQ3* insertion (SALK 098881) are listed in Supplemental Table 4 online.

Chlorophyll Extraction and Measurement

Tissue was snap frozen in liquid nitrogen and then chlorophyll was extracted using 80% acetone as previously described (Lichtenthaler, 1987). Absorbance was measured at 645 and 657 nm, and chlorophyll content was calculated using (20.2 × A645 + 8.02 × A657)/g fresh weight.

Yeast Two-Hybrid Assay

The Matchmaker GAL4 two-hybrid system (Clontech) was used for the yeast two-hybrid assay. The pGBT9 and pGAD424 vectors were used for making DNA binding domain and activation domain fusion constructs. The open reading frames of each gene were amplified from cDNA using gene-specific primers and inserted into pGBT9 and pGAD424 vectors using *Eco*RI and/or *Bam*HI restriction sites. Gene-specific primers are listed in Supplemental Table 6 online. β-Galactosidase liquid assays, for five colonies per construct, were performed using the protocol available at http://www.fhcrc.org/science/labs/gottschling/yeast/Bgal.html where

$$U = 1000 \times [(OD420) - (1.75 \times OD550)]/[(time) \times (vol) \times OD_{600}].$$

Phylogenetic Analyses

A total of 154 *Arabidopsis* bHLH sequences were identified based on BLAST searches and previously published data (Toledo-Ortiz et al., 2003). Full-length amino acid sequences were aligned using ClustalW (Thompson et al., 1994) with default values and refined by hand using MacClade 4.03 (Maddison and Maddison, 2000). The alignment is presented as Supplemental Data Set 1 online. Unrooted trees were generated using the neighbor-joining algorithm as implemented in PAUP 4.0 (Swofford, 2000) with default values. Bootstrap values for resolved nodes were derived from 1000 replicates using the neighbor-joining algorithm.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *AP3*, At3g54340; *PI*, At5g20240; *AST101*, At4g08620; *BNQ1* (*BHLH136*), At5g39860; *BNQ2* (*BHLH134*), At5g15160; *BNQ3* (*BHLH161*), At3g47710; *KIDARI*, At1g26945; and *BHLH135*, At1g74500. Accession numbers for all other sequences used are shown in Supplemental Figure 1 online.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Neighbor-Joining Analysis of 154 *Arabidopsis* bHLH Proteins.

Supplemental Figure 2. Digital Expression Profiling of *BNQ1*, *BNQ2*, and *BNQ3*.

Supplemental Figure 3. In Situ Expression Analyses of *BNQ2.*

Supplemental Figure 4. Complementation Analysis of *bnq3*.

Supplemental Table 1. RT-PCR Primer Sequences.

Supplemental Table 2. ChIP-PCR Primer Sequences.

Supplemental Table 3. In Situ Probe Primer Sequences.

Supplemental Table 4. SALK Line and RNAi Line Primer Sequences.

Supplemental Table 5. Overexpression Line Primer Sequences.

Supplemental Table 6. Yeast Two-Hybrid Primer Sequences.

Supplemental Data Set 1. Text File of the Alignment Used for the Phylogenetic Analysis Shown in Supplemental Figure 1.

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