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

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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 proteins 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* genes 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, 1998).
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., 2003; 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.

**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 sepal 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).

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**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
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
expression of BQN2 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 BQN1 and BQN3 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, BQN1 expression is observed in the first whorl of sepals (Figures 3G and 3I). In stage 12 ap3-3 and pi-1 flowers, BQN1 expression is also found in the second-whorl organs (Figures 3H and 3J). To determine if BQN1 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 BQN1 is expressed in each whorl of sepals regardless of position (Figures 3K and 3L). Similarly, in ap3-3 and pi-1 mutant flowers, BQN3 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 BQN3 in the second whorl (Figures 3S to 3V). This repression is tissue specific and not whorl specific, since in ag-1 mutant flowers, BQN3 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 BQN1 and BQN3 in developing petals.

To determine if the AP3/PI heterodimer binds to the promoters of the BQN 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)6GG] (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 BQN1, BQN2, and BQN3 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. Immuneoprecipitated 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 3. In Situ Expression Analyses of BQN Family Members.
(A) to (F) Expression (indicated by purple color) of BQN1 in wild-type (Ler) 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 BQN1 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 BQN3 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 BQN3 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.
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

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**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.

**Figure 5.** BNQ3 T-DNA insertional mutation.

**Figure 6.** BNQ3 T-DNA insertional mutation.

**Figure 7.** BNQ3 T-DNA insertional mutation.
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 phytochrome 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; Castillo 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 represing, HFR1 activity. Furthermore, these data suggest that the BNQ gene products interact with other, presumably as yet uncharacterized, atypical bHLH proteins.

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
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; Srithar 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 SPOROCYTE-LESS, encoding a putative transcription factor required for

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

(A) to (E) Individual flowers of Ler (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.
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

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**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.

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**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.
seeding 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., 2008; Hornitschek et al., 2009).

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

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.

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

| BNQ1-AD/BNQ1-BD | +/− | −/− | −/− | +/− | +/− | −/− |
| BNQ2-AD/BNQ2-BD | +/− | −/− | −/− | +/− | +/− | −/− |
| BNQ3-AD/BNQ3-BD | +/− | −/− | −/− | +/− | +/− | −/− |
| PGAD424/PGBT9   | −/− | −/− | −/− | +/− | +/− | +/− |

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 posttranscriptional 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 3SS:AP3-GR, 3SS:AP3 and 3SS:PI-HA) are in the Ler background. Mutant lines were obtained from the ABRC (Ohio State University). The ap3-3 3SS:PI 3SS: AP3-GR line was a gift from Robert W. M. Sablowski (John Innes Centre, Norwich, UK) (Sablowski and Meyerowitz, 1998). The 3SS: 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 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 RNA 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 BamHI and XbaI and inserted into the binary vector p235 (Jenik and Irish, 2001), a derivative of pPZP221 (Hajdukiewicz et al., 1994) containing the 3SS 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 3SS:BNQ3 were transformed into bnq3 mutants, whereas Ler 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 6 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 $(20.2 \times A645 + 8.02 \times A657)/g$ 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 EcoRI and/or BamHI 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.fhrc.org/science/labs/gottschling/yeast/Bgal.html where

$$U = 1000 \times [(OD_{420}) - (1.75 \times OD_{550})] \times (time) \times (vol) \times OD_{600}.$$ 

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 are 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, At1g06820; BNQ1 (BHLH136), At5g39860; BNQ2 (BHLH134), At5g15160; BNQ3 (BHLH161), At3g17710; 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 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.

Supplemental Table 6. In Situ Probe Primer Sequences.

Supplemental Table 7. ChiP-PCR Primer Sequences.

Supplemental Table 8. RT-PCR Primer Sequences.

Supplemental Table 9. RNAi Line Primer Sequences.

Supplemental Table 10. SALK Line Primer Sequences.

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