Brassinosteroids Confer Tolerance to Plants under the Nitrogen (N) Starvation Stress by Enhancing Low-N Induced Anthocyanin Biosynthesis

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Statement

All experiment works reported in this thesis were performed by the author, except that the EMSA experiments were done by Dr. Li, Shuo (The Chinese University of Hong Kong) and the analysis of interaction domain in PAP1 using yeast two-hybrid test was done by Mr. Li, Qianfeng (The Chinese University of Hong Kong).

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Abstract

Brassinosteroids (BRs) are a group of steroidal plant hormones that not only promote plant growth but also increase plant tolerance to a wide range of biotic and abiotic stresses. However, it is still not known whether BRs are involved in plant resistance to nutrient stresses such as N, Pi or K limitations. In this study, we found that exogenous application of 24-epibrassinolide (24-eBR), a BR analog, could substantially increase the tolerance of Arabidopsis plants to the N starvation stress. Similarly, *bzr1-1D*, a mutant that harbors a dominant mutation in the positive BR signaling component BZR1, also showed increased resistance to N starvation. More interestingly, both 24-eBR treatment and the *bzr1-1D* mutation could enhance the accumulation of anthocyanins induced by N starvation. Further, we found that BZR1 interacts with PAP1, a key transcription factor controlling the anthocyanin biosynthesis in plants, and that the expression of several 'late' anthocyanin biosynthetic genes were promoted by both 24-eBR and *bzr1-1D* mutation. However, the BR-enhanced anthocyanin accumulation was shown to be specific to plant response to low N stress, as neither 24-eBR treatment nor *bzr1-1D* mutation had a positive effect on the low Pi-induced anthocyanin biosynthesis in Arabidopsis plants.

摘要

油菜素内酯（BRs）是一类天然的类固醇植物激素。它们不但能有效促进植物的生长，还能显著提高植物对多种生物或非生物逆境的抗性。然而，到目前为止，人们对 BR 是否影响植物对各种营养胁迫（例如缺氮，缺磷或缺钾）的抗性，仍一无所知。我们发现，外源使用 24-表油菜素内酯 (24-eBR)（一种 BR）可以显著地提高模式植物拟南芥对氮亏缺的耐受性。同样， BR 信号传导途径的正调控因子 BZR1 的功能获得型突变体 *bzr1-1D*，对缺氮胁迫的耐受能力也很高。与此同时，在缺氮环境下， 24-eBR 预处理和 *bzr1-1D* 突变体还能明显增加拟南芥体内花青素的积累。我们进一步发现 BZR1 蛋白能够与控制花青素合成的关键转录因子 PAP1 蛋白直接相互作用，并通过与一些花青素合成酶的基因启动子结合，来调控它们的表达和促进花青素的合成。然而， BR 对花青素合成的促进似乎只特异地出现在低氮条件下，因为在缺磷条件下，24-eBR 预处理及 *bzr1-1D* 突变均不能进一步增加花青素的积累。

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A special acknowledgement should be shown to Mr. Li Qianfeng and Dr. Li Shuo. Mr. Li Qianfeng did the experiments on the interaction domain analysis of PAP1 in this thesis. He also provided the plasmids pGBKT7-BIN2/BES1/BZR1 and pGADT7-PAP1C1/C2/N1/N2 used in yeast two hybrid tests, as well as the constructs BZR1-nYFP/cYFP and nYFP/cYFP-BZR1 used in BiFC analysis, while all the EMSA assays mentioned in this thesis were done by Dr. Li Shuo.

Many thanks to Professor Cathrine Lillo (University of Stavanger, Norway), she had kindly provided me the seeds of *pap2* and *gl3* mutants.

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Part 1 Introduction

1.1 Brassinosteriods (BRs) and BR signaling

Plant growth is a complex, yet highly organized process, determined by the genotype and influenced by environments. 'Chemical messengers' had been suggested to coordinate this process since 1880s, when Charles Darwin and his son observed the phototropic movements in the coleoptiles of canary grass (Darwin, 1981). This observation greatly promoted the discovery and identification of plant hormones or phytohormones, which are signal molecules produced within a plant, occurring in extremely low concentrations and are fundamental regulators of plant growth (Leyser, 1998). Phytohormones are usually synthesized in one part of the plant and then transported to target locations to regulate physiological processes, such as cell elongation, cell division, shoot branching, flowering or fruit ripening (Kieber and Ecker, 1993; Masucci and Schiefelbein, 1996; Blazquez *et al.*, 1998; Ongaro and Leyser, 2008; Chen *et al.*, 2009), and to response to environmental factors, like light, gravity, temperature, and inorganic nutrients (Roman *et al.*, 1995; Li *et al.*, 1996; Evans and Ishikawa, 1997; Titarenko *et al.*, 1997; Schmidt *et al.*, 2000; Kagale *et al.*, 2007). Decades before, it had been thought there were only five groups of phytohormones, namely auxins, gibberellins (GA), cytokinins (CK), abscisic acid (ABA) and ethylene (Leyser, 1998; Gray, 2004). Each of them can

regulate a diverse range of cellular and developmental processes in plants, for example, IAA (indole-3-acetic acid), an auxin, regulates cell division and expansion, vascular differentiation, lateral root development, and apical dominance (Berliner, 1981; Reinhardt *et al.*, 2000; Teale *et al.*, 2006; Dubrovsky *et al.*, 2008; Teale *et al.*, 2008; Su and Zhang, 2009), while at the same time multiple hormones often act integratively to influence a single process, such as both auxin and CK involved in the regulation of plant cell cycle, and BRs, auxin, as well as ethylene together controlling the growth of hypocotyls growth (Redig *et al.*, 1996; Trehin *et al.*, 1998; Bonhomme *et al.*, 2000; Rahman *et al.*, 2002; Gazzarrini and McCourt, 2003; Gray, 2004; De Grauwe *et al.*, 2005; Rock and Sun, 2005; Li *et al.*, 2006; Teale *et al.*, 2008; Alonso-Ramirez *et al.*, 2009; Moubayidin *et al.*, 2009; Kushwah *et al.*, 2011; Lewis *et al.*, 2011). Recent studies have added new members to the family of phytohormones, including brassinosteroids (BRs), jasmotic acid (JA), salicylic acid (SA) and strigolactone (Clouse, 1996; Sakurai and Fujioka, 1997; Thomma *et al.*, 1998; Umehara *et al.*, 2008; Chen *et al.*, 2009; Fonseca *et al.*, 2009; Vlot *et al.*, 2009; de Saint Germain *et al.*, 2010; An and Mou, 2011; Nahar *et al.*, 2011). BRs, as the sixth group of phytohormones have been extensively studied in the last 40 years (Clouse and Sasse, 1998; Clouse, 2001; Nakaya *et al.*, 2002; Mussig, 2005).

1.1.1 Discovery of BRs

The discovery of BRs in plants was started by the recognition that pollen extracts from the rape plant (*Brassica napus* L.), that was originally named 'brassins', can

promote the growth of bean seedlings (Mitchell *et al.*, 1970; Mitchell and Gregory, 1972). In 1979, to identify 'the active factor(s)' of brassins, 10 mg of crystals were isolated from about 227 kg of bee-collected rape pollens and then subjected to X-ray crystallographic analysis to determine the structure, which was found to be a steroidal lactone with an empirical formula of $C_{28}H_{48}O_6$ (MW = 480), named as 'brassinolide' (Grove *et al.*, 1979). After that, over 70 natural brassinolide analogs have been identified so far and are collectively called brassinosteroids (BRs) (Bajguz & Hayat, 2009). These BRs are a group of polyhydroxyl steroids containing a common 5α-cholestan skeleton and are classified as C_{27}, C_{28} or C_{29} BRs depending on the alkyl-substitution pattern of the side chain (Thompson *et al.*, 1981; Kim *et al.*, 1990; Antonchick *et al.*, 2004; Uesusuki *et al.*, 2004; Acebedo *et al.*, 2011). Among them, brassinolide, 24-epibrassinolide and 28-homobrassinolide (Fig. a) are the three major kinds of BRs used in scientific studies.

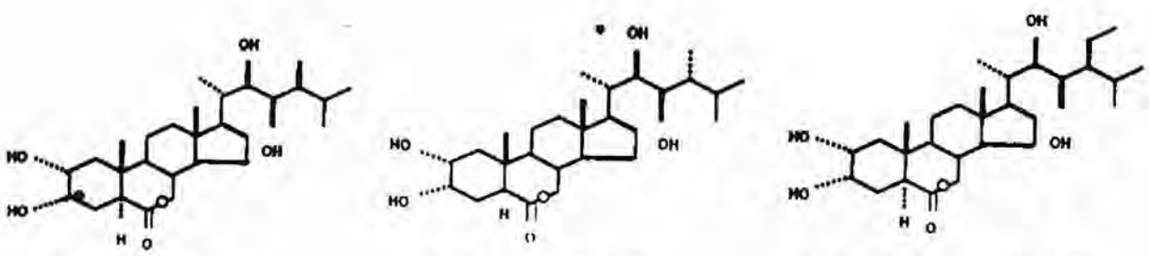


Figure a. Chemical structures of brassinosteriods

BRs have been demonstrated to be present in almost every part of plants at extremely low concentrations (nano-gram levels) (Sakurai and Fujioka, 1997; Shimada *et al.*, 2003). But the levels of endogenous BRs in young growing tissues are higher than that in mature tissues, when pollen and immature seeds contain the

highest level of BRs (Schmidt *et al.*, 1997).

1.1.2 Functions of BRs

BRs are plant hormones of great significance in plant growth and development (Brosa, 1999; Krishna, 2003; Mussig, 2005). This has been demonstrated by the characteristic phenotypes of BR-deficient and BR-insensitive mutants including dwarfism, curled leaves, male sterility, and light-grown morphology in the dark, and that the growth of BR biosynthetic mutants such as *cpd1* and *dwf1* was restored by exogenous application of brassinolide but not by gibberellin or IAA (Clouse *et al.*, 1996; Szekeres *et al.*, 1996; Fujioka *et al.*, 1997; Mathur *et al.*, 1998;Choe *et al.*, 1999; Noguchi *et al.*, 1999; Li *et al.*, 2001). What's more, the application of brassinozole (a specific inhibitor of BR biosynthesis) could result in dwarfism of *Lepidium sativum* plants, and the dwarfism was able to be reversed by exogenous application of brassinolide. All these indicated that BRs are essential to normal plant growth (Asami and Yoshida, 1999; Asami *et al.*, 2000).

Virtually, BRs are important regulators in various aspects of plant growth and development, such as cell elongation, cell division, senescence, vascular differentiation, reproduction, and photomorphogenesis (Szekeres *et al.*, 1996; Altmann, 1998; Clouse and Sasse, 1998; Brosa, 1999; Bishop and Yokota, 2001; Ye *et al.*, 2010). The failure in normal cell elongation in the BR-deficient *dwf4* mutant could be entirely and specifically rescued by exogenously supplied brassinolide, indicating that BR was an absolute requirement for cell elongation (Azpiroz *et al.*,

1998). The BR-increased cell division was observed in cultured parenchymatous cells of *Helianthus tuberosus*, leaf protoplasts of *Petunia hybrida* and protoplasts of Chinese cabbage (Hu *et al.*, 2000; Gonzalez-Garcia *et al.*, 2011; Bezrukova *et al.*, 2002). BRs also promoted the hypocotyl elongation in Packchoi (*Brassica chinensis*) with an increase in wall relaxation properties and a passive dilution of the osmotic pressure of the cell sap (Wang *et al.*, 1993). BR-deficient Arabidopsis mutants exhibited delayed chloroplast senescence, indicating that BR might play a role in regulating the processes of senescence (Li *et al.*, 1996), which was confirmed by the study of BRs on the ripening of tomato pericarp discs. Application of BRs to tomato pericarp discs increased the lycopene levels while decreased the chlorophyll levels. In addition, an increase in ethylene production was associated with BRs-accelerated fruit-senescence (Vidya Vardhini and Rao, 2002).

Furthermore, BRs were also found to increase plant resistance to various environmental stresses, such as heat, cold, drought, and salinity (Krishna, 2003; Kagale *et al.*, 2007; Kim *et al.*, 2010). A study of Kagale *et al.* (2007) showed that the treatment of 24-epibrassinolide (24-eBR) increased the basic thermotolerance of Arabidopsis, *Brassica napus* and tomato seedlings, which was, at least partly, due to the higher accumulation of the major classes of heat shock proteins (hsps) in 24-eBR-treated seedlings as compared with untreated seedlings after heat stress. This was related to higher levels of several translation initiation and elongation factors (eIFs and eEFs) in 24-eBR-treated seedlings, which meant that BRs functioned to protect the translational machinery under thermal stress (Dhaubhadel *et al.*, 1999;

Dhaubhadel *et al.*, 2002; Singh and Shono, 2003). BRs can also enhance drought tolerance of *A. thaliana* and *B. napus*. After 72-h drought stress, about 80% of BR-treated *A. thaliana* seedlings compared to only 35 % of untreated seedlings survived and continued to grow; similarly 90% of BR-treated *B. napus* seedlings and 20% untreated seedlings were recovered after 36 h of drought treatment. The BR-increased tolerance to drought was also confirmed by the higher levels of drought responsive genes (*rd29A*, *ERD10 etc.*) in BR-treated *A. thaliana* seedlings at the earlier time points of stress (up to approximately 60 h of stress) (Kagale *et al.*, 2007). In addition, BR also helps to overcome low germination rates of *B. napus* under high salt conditions. The presence of 2 μM 24-eBR can increase the average germination rate of *B. napus* seeds from 3% (control) to 34% (BR-treated) under the treatment of 300 mM NaCl (Krishna, 2003). Moreover, BR can restore the level of chlorophylls and increase nitrate reductase activity under salt stress (Divi *et al.*, 2010). However, whether BRs or BR signaling are involved in plant nutrient stresses, such as N limitation or Pi starvation, is still unknown.

1.1.3 BR signaling pathway

Genetic and molecular studies in the model plant *Arabidopsis thaliana* have greatly advanced our understanding of the BR signaling pathways in plants. The current model suggests that BR is perceived by the cell-membrane localized BRI1 (BR-INSENSITIVE 1) receptor-like kinase, leading to the phosphorylation of BSK1 (BR-SIGNALING KINASE 1), another membrane-associated protein kinase. The

phosphorylated BSK1 then activates BSU1 (*bri1* SUPPRESSOR 1), a protein phosphatase, to inactivate the GSK3-like kinase BIN2 (BR-INSENSITIVE 2) - a negative regulator of BR signaling that inhibits BR signaling by phosphorylating and destabilizing two downstream transcription factors BZR1 (BRASSINAZOLE RESISTANT 1) and BES1 (BRI1-EMS SUPPRESSOR 1) (Li and Chory, 1997, Li *et al.*, 2001, Peng *et al.*, 2008b, Tang *et al.*, 2008, Ryu *et al.*, 2010). As a result, BR signal dephosphorylates and accumulates BZR1 and BES1 in nucleus, where they bind the promoters of BR responsive genes or interact with other transcription factors to trigger various BR responses (He *et al.*, 2002; Wang *et al.*, 2002; Yin *et al.*, 2002; Mora-Garcia *et al.*, 2004; He *et al.*, 2005; Li *et al.*, 2010).

Genetic studies of *bri1* mutant showed its insensitivity to BRs but it was still sensitive to auxins, cytokinins, ethylene, abscisic acid, and gibberellins, suggested that *BRI1* gene was critical to BR perception and signal transduction (Clouse *et al.*, 1996; Li and Chory, 1997). BRI1 had then been shown to have the ability to bind BRs by several biochemical and molecular experiments, for example, binding of immunoprecipitated BRI1 with radio-labeled brassinolide, the most active BR (He *et al.*, 2000; Wang *et al.*, 2001). In addition, *BRI1* also played a role in the homeostasis of endogenous BR levels as the *bri1* mutants accumulated much higher levels of biologically active BRs than wild-type plants (Noguchi *et al.*, 1999). Besides BRI1, two BRI1 homologs (BRL1 and BRL3) also have the ability to bind BRs, and genetic studies showed that they were partially redundant with BRI1 and of great importance in vascular development (Cano-Delgado *et al.*, 2004; Zhou *et al.*, 2004).

BIN2, a negative regulator in BR signaling, was identified in the screening for BR-insensitive mutants (Li *et al.*, 2001). Gain-of-function in BIN2 displayed the dwarf phenotype similar to *bri1* mutants, while loss-of-function in BIN2 displayed a phenotype of constitutive BR responses (Yan *et al.*, 2009). The accumulation of BIN2 protein is decreased by BR treatment through the proteasome-mediated degradation pathway, as the BR-decreased BIN2 protein could be reversed by the treatment of 26S proteasome inhibitor MG132 (Peng *et al.*, 2008b). Further studies have shown that BIN2 was directly regulated and dephosphorylated by a Kelch-repeat containing protein phosphatase, BSU1 (Kim *et al.*, 2009).

The BSU1 phosphatase identified from a *bri1*-suppressor mutant screen plays a positive role in BR signaling pathway and acts downstream of BRI1 (Kim *et al.*, 2009). Studies have shown that BSU1 binds and dephosphorylates BIN2 at Y200 (a conserved phospho-tyrosine residue) to inhibit BIN2 function and leads to the accumulation of dephosphorylated BZR1 and BES1 proteins in the nucleus (Kim *et al.*, 2009).

BZR1 and BES1 are two transcription factors belonging to a small family of plant unique proteins, and mediate diverse BR responses (He *et al.*, 2002; Wang *et al.*, 2002; Yin *et al.*, 2002; Zhao *et al.*, 2002; Li and Deng, 2005,). They are close homologs with 88% identical sequence and both have three major domains: DNA binding domain, BIN2 phsophorylation domain (over 20 putative BIN2 phosphorylation sites), and a C-terminal domain (Wang *et al.*, 2002; Yin *et al.*, 2002; Zhao *et al.*, 2002; He *et al.*, 2005). Both BZR1 and BES1 accumulate in the nucleus

in response to BRs and trigger different BR-responsive gene expressions (Wang *et al.*, 2002; Yin *et al.*, 2002).

A genetic study of a mutant that is resistant to the BR-biosynthesis inhibitor brassinazole (brassinazole resistant 1, *bzr1-1D*) led to the identification of BZR1 (Wang *et al.*, 2002). The dominant *bzr1-1D* mutation stabilizes BZR1 protein, causes plant insensitivity to the BR biosynthetic inhibitor brassinazole (BRZ) and partially suppresses every aspect of the *bri1* phenotype, including cell elongation, leaf color and fertility. This indicates that BZR1 plays a positive role in BR signaling. However, in the light-grown *bzr1-1D* mutant, levels of BR and expression of *CPD* (a BR biosynthetic gene that is feedback inhibited by BR signaling) are both reduced, suggesting a negative effect of BZR1 on BR biosynthesis (Szekeres *et al.*, 1996; Mathur *et al.*, 1998; Wang *et al.*, 2002). Further study has found that BZR1 could directly bind to the promoters of BR biosynthetic genes which have a conserved BR response element (BRRE) [CGTG(T/C)G], and leads to the feedback inhibition of BR biosynthesis (He *et al.*, 2005).

The screening for suppressors of *bri1-119* (a weak allele of *bri1*) resulted in the identification of BES1 (*bri1*-EMS-suppressor 1) (Yin *et al.*, 2002). The dominant mutation of *bes1-D* is the same conserved proline changed to leucine as in *bzr1-1D*, results in the stabilized BES1 protein, a phenotype of constitutively activated BR-pathway, and fully suppression of the *bri1* mutation. BES1 has been shown to bind to E box (CANNTG) sequences present in many BR-induced gene promoters and activates BR target gene both *in vitro* and *in vivo* (Yin *et al.*, 2005). However,

unlike BZR1, BES1 has no feedback regulation on BR biosynthesis.

Recently, thousands of genes have been identified as direct targets of BZR1 and BES1 by chromatin immunoprecipitation followed by genomic tiling array (ChIP-chip) (Sun *et al.*, 2010; Yu *et al.*, 2011). From the analysis of promoter elements, it seems that both BZR1 and BES1 can bind to both BRRE and E-boxes (particularly G-box sequence: CATGTG and CACGTG) *in vivo*. Meanwhile, BRRE and E-box are also enriched in both BR-induced and BR-repressed gene promoters (Sun *et al.*, 2010; Yu *et al.*, 2011). This suggests that additional promoter sequence elements flanking BRRE or E-boxes or other interacting factors may be involved in the activation or repression of BZR1 and BES1 target genes.

To summarize, BRs are a group of steroidal plant hormones that are not only essential in regulating various aspects of plant growth and development, but also increase plant tolerance to a wide range of biotic and abiotic stresses. The BR signal is perceived by BR receptor-BRI1, and then transduced by BSK1, BSU1, and BIN2 into nucleus, where the two transcription factors BZR1 and BES1 are dephosphorylated and accumulated to mediate diverse BR responses.

1.2 Nitrogen (N) and N responses

Nitrogen (N) is a required mineral nutrient of plants, which constitutes 1.5–2% of plant dry matter and is a key component of essential biomacromolecules including proteins, nucleic acids, and chlorophyll (Arnon and Stout, 1939). Both inorganic forms (such as nitrate or ammonium) and organic forms (like free amino acids) of N,

are able to be absorbed from the soil, but the major form is nitrate (Lejay *et al.*, 1999; von Wiren *et al.*, 2000; Gojon *et al.*, 2011). However, N availability in soil fluctuates a lot and often limits plant growth and development. In order to optimize their nutrition, plants have developed an efficient sensing and response system to external and internal N status, including the regulation of nitrogen uptake capacity (Gazzarrini *et al.*, 1999; Lejay *et al.*, 1999; Cerezo *et al.*, 2001; Gansel *et al.*, 2001; Krouk *et al.*, 2006; Loque *et al.*, 2006; Wirth *et al.*, 2007; Gojon *et al.*, 2009), remobilization of N from old organs to new ones (Dechorgnat *et al.*, 2011; Lin and Wu, 2004; Richard-Molard *et al.*, 2008), changes in root architecture (Forde, 2002a; Boukcim *et al.*, 2006; Mantelin *et al.*, 2006; Walch-Liu *et al.*, 2006; Mi *et al.*, 2008), adjustment of shoot/root growth balance (Stadenberg *et al.*, 1994; Richard-Molard *et al.*, 1999; Huo *et al.*, 2008), and the accumulation of the pigments anthocyanins (Lea *et al.*, 2007; Peng *et al.*, 2008a; Tanaka *et al.*, 2008; Akerstrom *et al.*, 2009; Han *et al.*, 2010; Lovdal *et al.*, 2010).

1.2.1 Hormones involved in plant N responses

Studies have shown that phytohormones are also involved in plant N responses (Kiba *et al.*, 2011). For example, cytokinins (CKs) have been proposed to function as long-distance signals to control nitrogen assimilation and status in plants. It is known that increasing nitrate supply through the roots, but not the shoot, is able to induce expression of nitrate responsive genes in leaves. The fact that adding CK to plants can mimick this response, suggests that CK may act as long-range messengers,

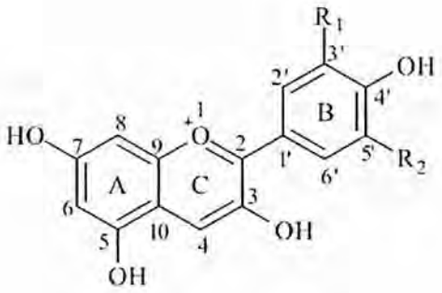
traveling from root to shoot, to control nitrate responses. In addition, CK biosynthesis is increased by the application of nitrate and CK is able to be transported through the vascular vessels, which all support this hypothesis (Sugiharto *et al.*, 1992; Forde, 2002b; Takei *et al.*, 2002; Sakakibara, 2003; Takei *et al.*, 2004; Sakakibara *et al.*, 2006; Hirose *et al.*, 2008). It was also observed that ABA-deficient or ABA-insensitive mutants in Arabidopsis showed lower inhibition of lateral root elongation after nitrate application, indicating that ABA mediates, at least in part, nitrate-triggered lateral root inhibition (Schraut *et al.*, 2005; Liang *et al.*, 2007; Zhang *et al.*, 2007; Planchet *et al.*, 2011). Furthermore, it was found that levels of auxins were higher in plant roots grown under low-nitrate conditions compared with plants grown under high-nitrate conditions, however, this was reversed in in nitrate-deprived shoots (Walch-Liu *et al.*, 2006; Tian *et al.*, 2008). This altered pattern of auxins accumulation in root and shoot in according to N conditions might indicate that auxin played a role in the adjustment of shoot/root growth balance to N availability.

However, to date, it remains unknown whether BRs play any role in plant N responses. This study provides evidence that BR is involved in plant responses to N deficiency, particularly in the low N-induced anthocyanin accumulation in Arabidopsis.

1.3 Anthocyanin and anthocyanin synthesis

1.3.1 Anthocyanin structures

Anthocyanins are a branch of flavonoids, naturally occurring in all tissues of higher plants as glycosides, having glucose, galactose, rhamnose, xylose or arabinose attached to an aglycon nucleus (Hungria *et al.*, 1991, Choung *et al.*, 2003). They are water-soluble vacuolar pigments, which may appear in red, purple, or blue color depending upon the pH of solvent and the presence of chelating metal ions. The sugar-free counterparts of anthocyanins are known as anthocyanidins. The most common skeletons of anthocyanidins are cyanidin, delphinidin, pelarogonidin, malvidin, petunidin, and peonidin (Fig. b). Different sugar components are conjugated to the basic anthocyanidin skeletons to form hundreds of anthocyanins with various positions and extent of glycosides attached to the skeletons (Bloor and Abrahams, 2002).



Name	R1	R2
Delphinidin	OH	OH
Petunidin	OCH_3	H
Cyanidin	OH	H
Pelargonidin	H	H
Peonidin	OCH_3	H
Malvidin	OCH_3	OCH_3

Figure b. Chemical structure of anthocyanidins

1.3.2 Functions of anthocyanins

As an important class of secondary metabolites, anthocyanins have diverse functions. They provide the attractive colors in flowers to recruit pollinators and in fruits to draw animals to disperse seeds (Hoch *et al.*, 2001; Diaz *et al.*, 2006), and are induced by various biotic and abiotic stresses such as pathogen attack, wounding, low temperature, UV light, and nutrient stress (Noh and Spalding, 1998; Hoch *et al.*, 2003; Reyes and Cisneros-Zevallos, 2003; Rowan *et al.*, 2009) to help plants survive from the changing environments (Peng *et al.*, 2008a). Besides their numerous functions in plants, anthocyanins also function as 'nutraceutical' compounds with plenty of medicinal, pharmaceutical, and nutritional properties to prevent several diseases including cancer (Mazza, 2007; Saluk-Juszczak, 2010). They exhibit antioxidant activity, induce apoptosis, stimulate DNA repair, and inhibit the division of cancer cells (Netzel *et al.*, 2006; Jing *et al.*, 2008; Ginjom *et al.*, 2010; Zhang *et al.*, 2011).

1.3.3 Biosynthesis of anthocyanins

Anthocyanins are synthesized via a branch of the phenylpropanoid pathway in plants, which is subdivided into 'early' steps and 'late' steps. In Arabidopsis, the 'early' step genes include *chalcone synthase* (*CHS*), *chalcone-flavanone isomerase* (*CHI*) and *flavanone 3-hydroxylase* (*F3H*), while the 'late' step genes are *flavanone 3'-hydroxylase* (*F3'H*), *dihydroflavonol 4-reductase* (*DFR*), *anthocyanidin synthase* (*ANS*), *anthocyanin glycosyltransferase* (*AGT*), and *glutathione S-transferase* (*GST*)

(Fig. c) (Winkel-Shirley, 2001). The classification of 'early' and 'late' genes essentially depends on whether the genes are regulated by the WD-repeat /bHLH/Myb transcriptional complex (Gonzalez *et al.*, 2008).

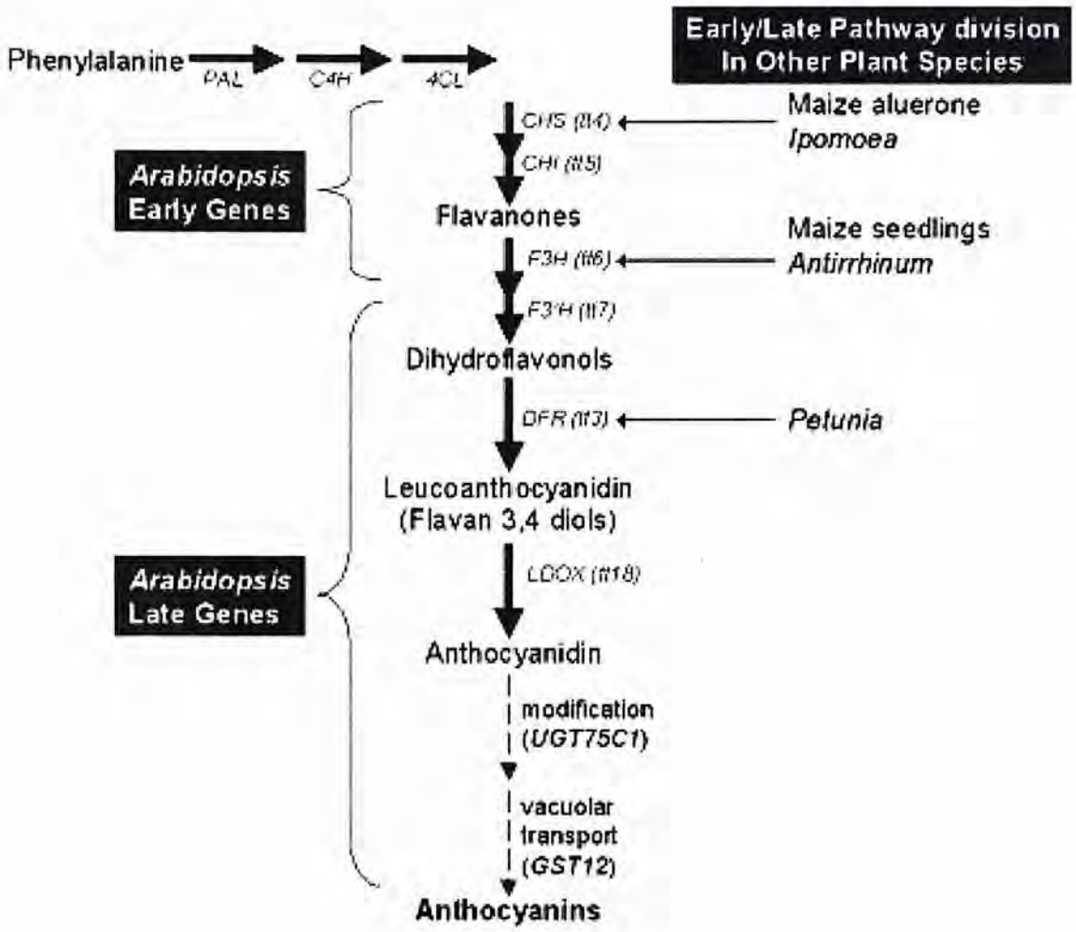


Figure c. The branch of the phenylpropanoid biosynthetic pathway yielding anthocyanins.

Brackets indicate the early and late divisions of the anthocyanin synthetic pathway in Arabidopsis; Thin horizontal arrows indicate the early and late divisions of anthocyanin synthetic pathway in other plant species. (From Gonzalez *et al.*, 2008)

1.3.4 Regulation of anthocyanin biosynthesis

The WD-repeat/bHLH/Myb complex is a combination of three major types of transcription factors, including members of the WD-repeat (Transparent Testa

Glabra1, TTG1) , bHLH (such as Transparent Testa 8, TT8; Glabra 3, GL3 and Enhancer of Glabra 3, EGL3) and Myb (such as Production of Anthocyanin Pigment1, PAP1; Production of Anthocyanin Pigment 2, PAP2; MYB113 and MYB114) protein families, and controls both the developmental and stress-responsive anthocyanin biosynthesis (Quattrocchio *et al.*, 1998; Borevitz *et al.*, 2000; Nesi *et al.*, 2001; Gonzalez *et al.*, 2008; Gonzalez *et al.*, 2009; Yuan *et al.*, 2009).

TTG1 is a WD40 repeat protein regulating several characters in Arabidopsis, including the formation of hairs on leaves, stems, and roots, and the production of anthocyanin pigments (Schnittger *et al.*, 1999; Walker *et al.*, 1999; Bouyer *et al.*, 2008; Maes *et al.*, 2008). It is proven by the phenotypes of the *ttg1* mutants that are glabrous, possessing none of the leaf or stem hairs (trichomes) and completely lack anthocyanins in the epidermis and in subepidermal layers of leaves, stems and seed coats (Walker *et al.*, 1999). Further, in *ttg1* mutants, the anthocyanin biosynthetic pathway is blocked at the dihydroflavonol-4-reductase (*DFR*) step, as transcripts of *DFR* have not been detected in the mutants contrast to the transcripts of the *CHS* and *CHI* genes which are not affected (Shirley *et al.*, 1995). TTG1 interacts with the bHLH transcription factors GL3, EGL3 and TT8 as shown in yeast two-hybrid assays, as well as the R2R3 MYB transcription factors PAP1, PAP2 and TT2, to form the WD-repeat/bHLH/Myb complex and to regulate the target biological processes (Broun, 2005). *TTG1* as a conserved regulator within plant kingdom, playing a central role in the production of anthocyanin and other secondary metabolism

processes, is constitutively expressed, and is not influenced by nutrient deprivation in Arabidopsis (Lillo *et al.*, 2008).

PAP1 (MYB75) is a R2R3 MYB transcription factor that positively regulates anthocyanin production by promoting the expression of 'late' anthocyanin synthetic genes (Dare *et al.*, 2008). Transgenic plants of PAP1 over-expression exhibited the purple phenotype as a result of anthocyanin accumulation (Borevitz *et al.*, 2000), while the *pap1* null allele failed to show the anthocyanins induced by sucrose in Arabidopsis hypocotyls as do in wild-type plants (Solfanelli *et al.*, 2006). These genetic studies suggested an important role of PAP1 in the regulation of anthocyanin biosynthesis in plants. Furthermore, the 'late' genes *DFR, GST, ANS* and *UFGT* (*UDP flavonoid 5-O-glycosyltransferase*) have been found to be directly regulated by PAP1 through a conserved PAP1 *cis*-regulatory element (C/T)CNCCAC(A/G)(A/T)(G/T) in their promoters (Dare *et al.*, 2008), which provides a direct evidence regarding the regulation of PAP1 on anthocyanin synthesis.

PAP2 (MYB90) is a homolog of PAP1 and also has similar functions in the regulation of the flavonoid pathway (Borevitz *et al.*, 2000). The levels of *PAP1* and *PAP2* transcripts are both increased in response to N or Pi deficiency, high intensity light and sucrose exposure (Lea *et al.*, 2007; Lillo *et al.*, 2008). But one of the differences between *PAP1* and *PAP2* is that the expression level of *PAP2* is extremely low under non-stressful conditions as compared with *PAP1*.

GL3 and **EGL3** are two bHLH transcription factors, both known to interact with

the MYB transcription factors PAP1 and PAP2 (based on the R3 repeat of the MYB domain) to activate the promoter of *DFR* gene (Zimmermann *et al.*, 2004). Furthermore, they can replace each other biochemically in protein interaction studies, and also physiologically when ectopically expressed. The anthocyanin structure genes have been demonstrated to be differentially regulated by GL3 and EGL3: *F3'H* is controlled by both *GL3* and *EGL3*, while *DFR* and *ANS* are much stronger affected by *EGL3* than *GL3*. *GL3* has been shown to be a necessary component in the induction of anthocyanin biosynthesis in the response to N deficiency. The transcripts of *GL3* as well as *PAP1*, *PAP2* were all strongly enhanced by N depletion (Lea *et al.*, 2007). In addition, N-deprivation induced anthocyanins were absent in cotyledons and rosette leaves of the *gl3* mutant. By contrast, the wild-type and *egl3* mutant both normally accumulated anthocyanins in response to N depletion in these experiments (Feyissa *et al.*, 2009). That means, endogenous *GL3* was not replaced by *EGL3* or other bHLH transcription factors in leaves to induce anthocyanins under N deprivation stress.

It is supposed that various combinations of different regulators in the WD-repeat/bHLH/Myb complex may specifically regulate the structural genes and response to different kinds of stimuli (Quattrocchio *et al.*, 1998; Nesi *et al.*, 2001). For example, in *Antirrhinum,* three MYB-related transcription factors were shown to regulate the anthocyanin structural genes with different specificity, which results in different patterns and intensity of anthocyanin pigmentation in *Antirrhinum* flowers (Schwinn and Venail, 2006).

1.4 Hormones and plant nutrient stresses

Studies have supported the involvement of phytohormones in nutrient-stress responses. The GA-DELLA signaling has been proved to mediate multiple Pi starvation responses (PSR), including alteration of root architecture, reduction of shoot growth, and accumulation of anthocyanins (Jiang *et al.*, 2007). CK can repress the expression of genes responsive to various nutrient starvation stresses (N, Pi, S, Fe) (Ohkama *et al.*, 2002; Franco-Zorrilla *et al.*, 2005; Wang *et al.*, 2006; Camacho *et al.*, 2008; Seguela *et al.*, 2008; Criado *et al.*, 2009) and negatively regulates PSR in a CK-receptor-depend way (Franco-Zorrilla *et al.*, 2002; Franco-Zorrilla *et al.*, 2005), while ethylene signaling is shown to be involved in the low-iron-induced root hair (Schmidt *et al.*, 2000).

Nevertheless, to date no clear evidence points to the involvement of BR signaling in nutrient-stress responses (Rubio *et al.*, 2009). Thus, in this study, we report that BR can protect plants from N starvation stress and this effect is positively correlated with BR-induced enhancement of anthocyanin biosynthesis under N starvation. Further mechanistic studies suggest that BR-induced anthocyanin accumulation is mediated by the interaction between BZR1, a transcription factor in BR signaling pathway, and PAP1, a key transcription factor in the WD-repeat/bHLH/Myb transcriptional complex that regulates anthocyanin biosynthesis in Arabidopsis.

Part 2 Materials and Methods

2.1 Plant materials and growth conditions

The materials used include three *Arabidopsis thaliana* ecotypes Columbia (Col), Wassileskija (Ws), and Enkheim-2 (En2); BR signaling mutants *bri1-5*, *bin2*, *bzr1-1D* and *bes1-D;* BR-deficient mutant *det2*; *pap2* and *gl3*. Of them, *bin2* (Li *et al.*, 2001), *bzr1-1D* (Wang *et al.*, 2002), *det2* (Fujioka *et al.*, 1997) and *pap2* (PAP2 T-DNA insertion mutant) (Alonso et al., 2003) are in Col-0 background , whereas *bri1-5* is in Ws background (Noguchi *et al.*, 1999); *bes1-D* is in En2 background (Yin *et al.*, 2002) and *gl3* (GL3 T-DNA insertion mutant) is in Ler background (Feyissa *et al.*, 2009). All seeds were surface-sterilized with 70% ethanol + 0.1% Triton X-100 for 10 min, washed twice with 95% ethanol for 2 min, and finally dried on sterilized filter papers in a laminar hood. The seeds were then spread on Murashige and Skoog (MS) agar midia supplemented with 1% sucrose, and kept at 4 °C in dark for 3 days to synchronize germination before being transferred to a growth chamber for germination (22 °C, with a 16h/8h light/dark cycle). The seeds were grown for 5 days in a growth chamber before subjecting to further treatments.

For the recovery experiment of low-N stressed plants, 5-day-old Arabidopsis seedlings grown on MS agar plates with 10^{-10} mol/L 24-epibrassinolide (24-eBR) or $1/10^{8}$ ethanol (v/v) (mock) were transferred to N-free MS media for up to 40 days of N deprivation. The N-deprived seedlings were then carefully transferred to normal MS agar plates to allow the plants to recover from N deprivation. The recovery rate

of each genotype of plants was recorded.

For all other experiments (such as measurement of anthocyanins and real-time PCR analysis), 5-day-old Arabidopsis seedlings grown on normal MS agar plates were pretreated with 10^{-6} mol/L 24-eBR or $1/10^4$ ethanol (v/v) (mock) for 0.5 hour before they were transferred to N-free medium for 14 days (anthocyanin measurement) or 10 days (real-time PCR).

For nitrate treatment, 5-day-old Arabidopsis seedlings (wild-type Col-0 and transgenic plants *CPDpro::GUS*) were transferred to grow on MS medium with different nitrate (NO_3^-) concentrations for 8 days.

2.2 Measurement of anthocyanin content

Arabidopsis seedlings were pretreated with 10^{-6} mol/L 24-eBR for 0.5 hour before they were exposed to different nutrient stresses: N, potassium (K), or phosphorus (Pi) starvation. After 14 day's stress treatment, the materials were collected for anthocyanin measurements following the procedure of Kim *et al.* (2003). The anthocyanins were extracted by incubating 50 mg of shoot tissues in 300 μL of extraction solution (methanol plus 1% HCL, v/v) overnight at 4 °C in darkness. After the extraction, 200 μL of water and 200 μL of chloroform were added, and the mixture was centrifuged at 14,000 rpm for 2 min to remove the tissue debris. 70 μL of the supernatant solution was used to measure the anthocyanin content, which was detected by a Spectrophotometer (ENS) and calculated as (A530 -0.33*A657) per gram of fresh weight. The contents of anthocyanins for each sample were measured

with at least three independent biological replicates.

2.3 Yeast two-hybrid (Y2H) assay

The Y2H assay was carried out using the Matchmaker GAL4 system (Clontech, USA). Briefly, the assay is based on the principle that the eukaryotic gene transcription is controlled by transcription factors with two domains (such as GAL4), a DNA binding domain (BD) and a transcription activation domain (AD). These two domains need to be physically close to each other to start transcription. The BD domain functions to bind the upstream activating sequence (promoter) of the target genes, while the AD is responsible to activate the transcription of a downstream (reporter) gene. One protein ("bait") is tagged to the BD and another protein ("prey") is fused to the AD. They are then co-transformed into yeast. If there are protein-protein interactions between the bait and the prey proteins, AD and BD will be brought into close proximity. As a result, the transcription of the reporter gene ensues (Fig. d). The final step is to use suitable methods to select the positive colonies in which the reporter gene is expressed from the negative colonies (Zhu, 1997).

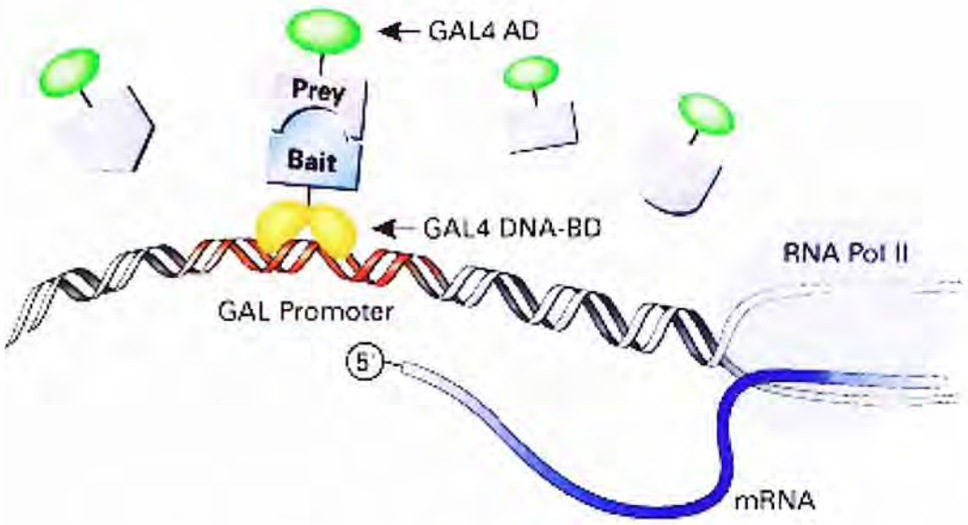


Figure d. Principle of the yeast two-hybrid test

A bait protein interacts with the GAL4 recognition sequence (or promoter) upstream of a reporter gene. Transcription of the reporter is activated when a prey protein containing the GAL4 transcriptional activation domain interacts with the bait. (From http://www.clontech.com)

In our experiments, the cDNAs encoding BZR1, BES1 and BIN2 were cloned into the pGBKT7 vector as the bait constructs, while the cDNAs encoding PAP1, PAP2, GL3 as well as the deleted PAP1 fragments were cloned into pGADT7 as prey constructs. The bait-prey construct pairs to be tested were co-transformed into the yeast strain AH109. Since BZR1, BES1, and BIN2 all have transcription-activation activity even when their bait constructs were transformed into yeast alone, the screening SD media for protein-protein interaction (-Ura/-His/-Trp/-Leu) using these three proteins as baits were supplemented with 50, 25, and 20 mM 3-amino-1,2,4-triazole (3-AT), respectively (He *et al.*, 2002).

2.4 Bimolecular fluorescence complementation (BiFC) assays

Bimolecular fluorescence complementation (BiFC) is a technique for visualization

of protein-protein interactions *in vivo*. The yellow fluorescent protein (YFP) was split into two non-fluorescent and non-overlapping N-terminal (nYFP) and C-terminal (cYFP) fragments. Each fragment was cloned in-frame to a gene of interest, enabling expression of fusion proteins. If the two tested protein interacted with each other, the YN and YC fragments would be brought together and formed a fluorescent complex, which could be detected by a confocal microscope (Fig. e) (Schutze *et al.*, 2009; Bracha-Drori *et al.*, 2004).

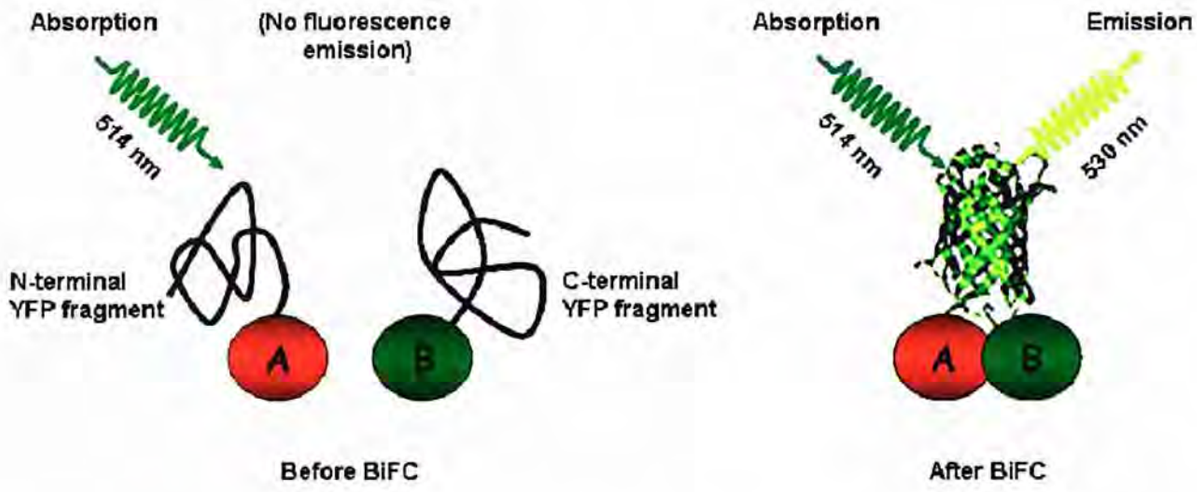


Figure e. Principle of the BiFC assay

Proteins A and B are fused to nYFP and cYFP fragments, respectively. In the absence of an interaction between A and B, the fluorophore halves remain non-functional. As a result of the interaction between A and B, a functional fluorophore is formed which exhibits emission of fluorescence upon excitation with an appropriate wavelength (From Bhat *et al.*, 2006).

According to the principle, cDNAs encoding Arabidopsis PAP1 and BZR1 were first cloned into the TOPO-entry vector of the Gateway Cloning System (Invitrogen) and then recombined into the binary BiFC vectors containing the yellow fluorescent protein (YFP) sequence to generate BZR1-nYFP, BZR1-cYFP, nYFP–BZR1,

cYFP-BZR1 and PAP1-nYFP, PAP1-cYFP, nYFP–PAP1, and cYFP-PAP1 fusions. These constructs were transformed into the *Agrobacterium tumefaciens* strain GV3101. *Agrobacterium* strains harboring the BZR1-PAP1 pairs in different nYFP-cYFP combinations were co-infiltrated into the *Nicotiana benthamiana* leaf for transient assay of the BZR1-PAP1 interaction (Qi *et al.*, 2011). Plants were placed at 24 ℃ for 48 h before detection of YFP fluorescence. YFP fluorescence was detected with a Confocal Microscope (Bio-Rad Radiance 2100) and analyzed by the FV10-ASW 1.7 Viewer software.

2.5 Quantitative real-time PCR

Col-0 and *bzr1-1D* seedlings were pretreated as mentioned and then grown under N starvation for 10 days. Total RNA was extracted from the whole seedlings with the Trizol RNA extraction kit (Invitrogen) according to the manufacturer's instruction, and then subjected to a DNase Ⅰ (Promega) digestion. One microgram of RNA was then reverse transcribed into cDNA. Real-time PCR amplification was carried out using the SYBR Green Supermix (Bio-Rad), and detected by Bio-Rad iQ™ 5 detection system with *UBC* as an endogenous control gene. Relative quantitation of expression level of each gene was performed using the comparative CT method as described previously (Li *et al.*, 2009). Primer pairs used for PCR amplification were presented in supplementary materials. The expression level of each gene was measured with three biological repeats.

2.6 Electrophoretic mobility shift assay (EMSA) and competition assay

Electrophoretic mobility shift assay (EMSA) is an important technique for studying protein:DNA interactions (Revzin, 1989; Ramanujam *et al.*, 1990). The technique is based on the fact that protein:DNA complexes migrate more slowly than free linear DNA fragments in a native polyacrylamide or agarose gel, resulting in a "shift" in migration of the labeled DNA band. There are three key steps in the gel shift assay: (1) setup of the binding reactions, (2) electrophoresis, and (3) probe detection.

In order to verify the specificity of the tested protein:DNA binding, a specific competitor is used as an important control. The specific competitor usually has the identical sequence as the labeled probe but not labeled. The unlabeled specific competitor must be added into the binding reaction before the labeled probe, to eliminate or reduce the positive shift results, due to the competition with the same labeled probe. While the mutant competitor is another important control to verifying the specific binding site in DNA sequence, the addition of excess mutant competitor with a low-affinity binding site will not compete with specific interactions, as a result the shifted band will be preserved (Fig. f) (Thermo Scientific instructions).

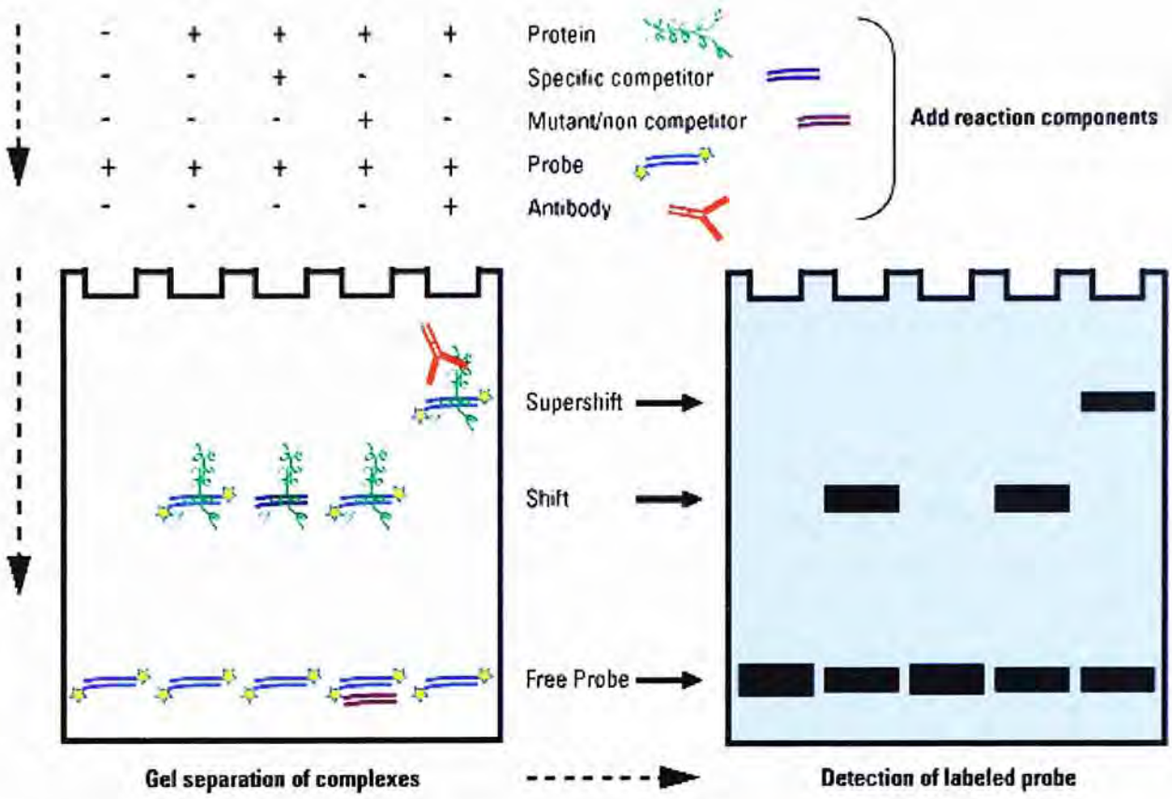


Figure f. Overview of the Gel Shift Assay Method (From http://www.piercenet.com/).

In our experiment, recombinant BZR1 proteins fused to the maltose binding proteins (MBP) were expressed and affinity-purified from *E. Coli* using amylose agarose beads. About 50-bp oligo probes containing the BRRE sequences used in EMSAs were chemically synthesized as listed in Table 1. EMSAs were performed according to the manufacturer's instruction (Biotin 3' End DNA Labeling Kit and LightShift ® Chemiluminescent EMSA Kit from Thermo Scientific). Each reaction contains 10 ng MBP-BZR1 protein and 2 ng probe DNA (He *et al.*, 2005).

Table 1. Probe sequences used in EMSA assays

DFR-F	caaactgaactgaagtcacc**cacacg**tctcaccaaacaaatcgaag
DFR-R	cttcgatttgtttggtgaga**cgtgtg**ggtgacttcagttcagtttg
PAP1-F	gtggatatcaaaca**tgcacg**tcacttccttttttccgtcacgtgttttta
PAP1-R	taaaaacacgtgacggaaaaaaggaagtga**cgtgca**tgtttgatatccac

PAP2-F	gatatcaaaca**tgcacg**tcattttcctatgcccttca**cgtgta**tatatatac
PAP2-R	gtatatata**tacacg**tgaagggcataggaaaatga**cgtgca**tgtttgatatc
mDFR-F	caaactgaactgaagtcacc**AAAAAA**tctcaccaaacaaatcgaag
mDFR-R	cttcgatttgtttggtgaga**TTTTTT**ggtgacttcagttcagtttg
mPAP2-F	gatatcaaaca**AAAAAA**tcattttcctatgcccttca**AAAAAA**tatatatac
mPAP2-R	gtatatata**TTTTTT**tgaagggcataggaaaatga**TTTTTT**tgtttgatatc

2.7 Histochemical staining of GUS activity

Whole seedlings of transgenic plants *CPD::GUS* grown on different nitrate conditions were submerged in staining solution consisting of 25 mM sodium phosphate buffer, pH 7.0, 2 mM 5-bromo-4-choro-3-indolyl-b-D-glucuronide cyclohexylamine salt (X-gluc), 0.5 mM ferricyanide, 0.5 mM ferrocyanide and 10 mM EDTA at 37℃ for 14 h. The GUS staining solution was then replaced with 70% ethanol for several times to bleach the tissues (Guo *et al.*, 2002).

Part 3 Results

3.1 24-epibrassinolide (24-eBR) increases plant tolerance to N-starvation in Arabidopsis

In order to understand whether BR and BR signaling play regulatory roles in plant N responses, the effects of BR and BR signaling on plant tolerance to N starvation were investigated by using wildtype (Col-0), BZR1 dominant mutant *bzr1-1D* and BR deficient mutant *det2* in *Arabidopsis*. 5-day-old seedlings were treated on N-free media for up to 40 days and then allowed for recovery on normal MS media. The recovery rate (the ratio of survived green plants to all plants tested) was recorded. As shown in Fig. 1, BR treatment significantly increased the seedling recovery rate after N deprivation stress. After 40 days' N deprivation, the mock treated wildtype plants (Col-0) only had 28.7% of recovery rate after being transferring back to normal +N medium for 6 days. However, this rate was increased to as high as 97.7% after the plants were pre-treated with 10^{-10} mol/L 24-eBR. Interestingly, the *bzr1-1D* mutant also had a recovery rate of 92.9% even without BR treatment; after BR pretreatment, its recovery rate reached 100% (Fig. 1). As BR treatment and the *bzr1–1D* mutation can both increase BZR1 protein accumulation and activity (Wang *et al.*, 2002), we inferred that that the BR-induced plant tolerance to N deprivation might be the result of increased BZR1 accumulation or activity. Consistently, the recovery rate of the BR deficient mutant *det2* was extremely low (4.4%) (Fig. 1) and the rate cannot be improved by 10^{-10} mol/L 24-eBR treatment.

It has been reported that BR increases plant tolerance to several abiotic stresses including cold, heat, drought and salinity. (Krishna, 2003; Kagale *et al.*, 2007; Kim *et al.*, 2010), however, to our knowledge, there is so far no report on the involvement of BR signaling in nutrient stresses. As we shown here, both endogenous and exogenous BR levels affect plant tolerance to N deprivation, which is likely through modulating the BZR1 protein accumulation and/or activity.

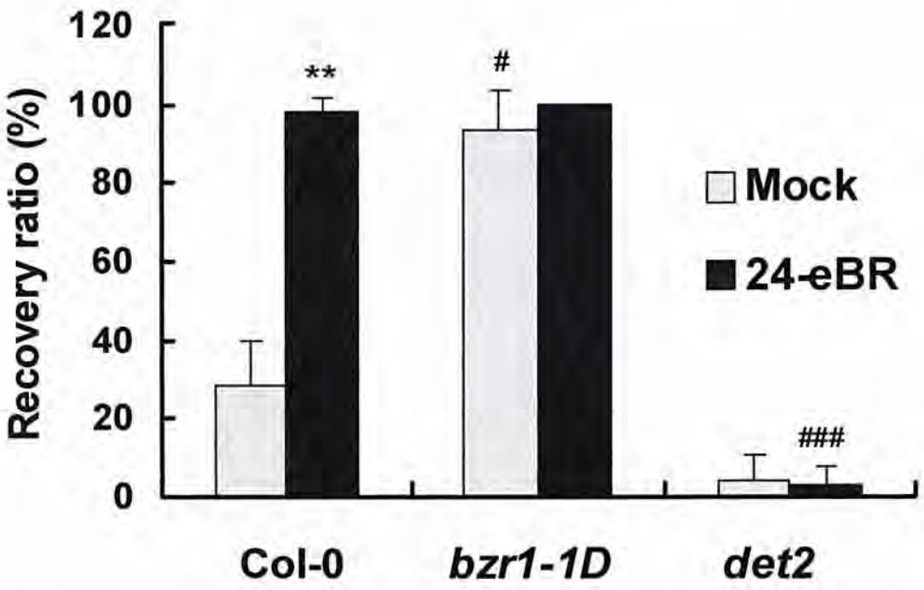


Figure 1. Effects of 24-epibrassinolide (24-eBR) on the recovery rate of N-deprived wild-type (Col-0) and BR-related mutants

5-day-old seedlings grown on MS medium supplemented with mock or 10^{-10} mol/L 24-eBR were transferred to N-free MS medium for N deprivation. After 40 days' N deprivation, the seedlings were recovered by N resupply to determine the recovery rate. Col-0, *Arabidopsis thaliana* ecotype Columbia; *bzr1-1D*, a BZR1 dominant mutant of *BZR1*; and *det2*, a BR deficient mutant.

* Stands for the significant difference between 24-eBR and mock treatment with other conditions the same.

Stands for the significant difference between BR mutants compared to Col-0 under the same treatment.

*/# P value< 0.05; **/## P value< 0.01; ***/### P value< 0.001

3.2 BR treatment enhances anthocyanin accumulation under N deprivation conditions

Under low N conditions, plants accumulate anthocyanins to protect them from N deficiency (Peng *et al.*, 2008a). In our study, in accordance with the BR-induced low N tolerance, we also found that 24-eBR enhanced anthocyanin accumulation in Arabidopsis seedlings under N deprivation, particularly in the *bzr1-1D* mutant. In order to minimize the side effects of long-time BR treatment, in all the following experiments we used the short-time BR treatment (10^{-6} mol/L 24-eBR for 0.5h). The results showed that 0.5 h of BR treatment significantly enhanced the anthocyanin accumulation in Col-0 after 14d's N deprivation , so did the *bzr1-1D* mutation, by which anthocyanin content was increased more than 50% compared to that of the mock-treated control (Fig. 2a&b). Again, as both BR treatment and the *bzr1–1D* mutation can increase BZR1 accumulation or activity, the above results indicated that the BR-enhanced anthocyanin production under N deprivation could be due to the increased BZR1 protein accumulation or activity in BR-treated plants. However, the anthocyanin content in *det2* is low and cannot be rescued by 0.5 h of 10^{-10}M BR treatment, suggesting that a higher BR level might be required to rescue the anthocyanin biosynthesis in *det 2* under N starvation.

To better understand the mechanisms of the BR-induced anthocyain accumulation, we further examined the anthocyanin contents in other BR signaling mutants under N deprivation, including *bri1-5* (a weak loss-of-function mutant of the BR receptor BRI1 in Ws background) and *bes1-D* (a dominant mutant for the

transcription factor BES1, in En2 background). BES1 and BZR1 are close homologs, sharing 88.9% amino acid sequence identity, and the *bzr1-1D* and *bes1-D* mutations result in the same amino acid change (Proline to Leucine) (Wang *et al.*, 2002; Yin *et al.*, 2002). But unlike *bzr1-1D*, the *bes1-D* mutant has similar anthocyanin content to its wild type En2 under either the normal or limiting N condition, and BR treatment cannot change this pattern, albeit the anthocyanin levels in both BR-treated En2 and *bes1-D* plants are higher (Fig. 2c). This in vast contrast to *bzr1-1D,* in which the anthocyanon level was much higher than its background Col-0 under N deprivation, and the BR treatment further increased its anthocyanin accumulation (Fig. 2b). What's more, the expression of BES1 was significantly inhibited by 10-day N deprivation stress, while the expression of BZR1 was slightly induced by N deprivation in wild-type Arabidopsis (data not shown). These interesting results indicated that in the BR signaling pathway, it is BZR1 rather than BES1 mediating the BR-induced anthocyanin accumulation under N deprivation condition.

In the case of *bri1-5,* 0.5 h of BR treatment failed to increase the anthocyanin content in seedlings even under N-deprivation condition, which is consistent with the fact that *bri1-5* is a BR-insensitive mutant (Fig. 2d). This means that BR-induced increase of anthocyanin biosynthesis under N deprivation requires a functional BR receptor. In mock (non-BR)-treated *bri1-5* plants, the anthocyanin level was similar to that in the mock-treated Ws control after 14d's N deprivation (Fig. 2d), suggesting that low-N-induced anthocyanin accumulation itself is independent of the BR receptor, or reflecting the fact that *bri1* mutants accumulate higher levels of BRs

(Noguchi *et al.*, 1999).

To summarize, under the N deprivation condition, exogenous BR treatment enhances anthocyanin accumulation in Arabidopsis seedlings. Both BR level and BR signaling affect N-deprivation-induced anthocyanin accumulation. The extremely high accumulation of anthocyanin in the *bzr1-1D* mutant suggests that BZR1 is a major regulator of BR-induced anthocyanin accumulation under N deprivation stress.

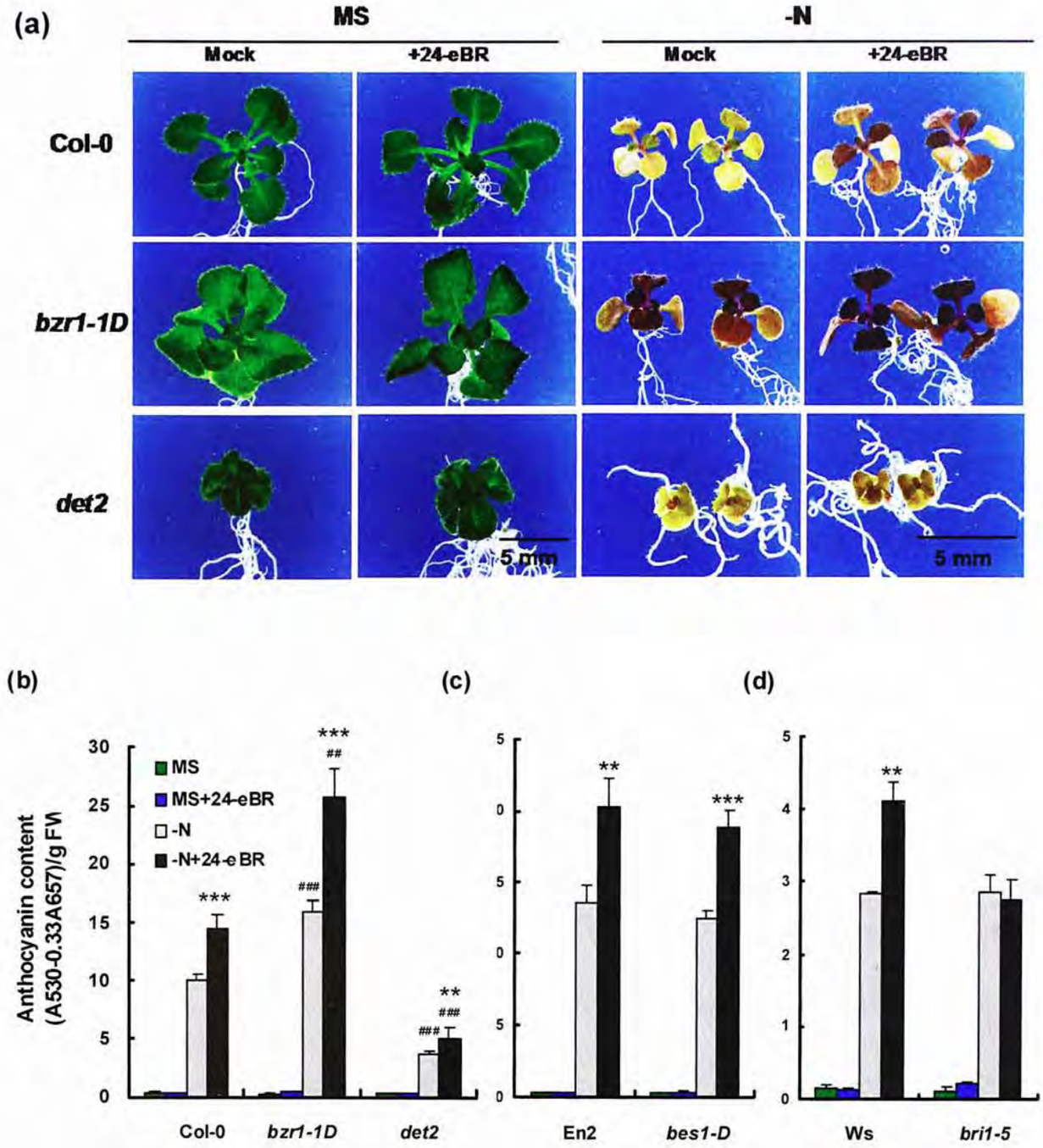


Figure 2. BR enhances anthocyanin accumulation in Arabidopsis under N deprivation

5-day-old seedlings grown on MS medium were treated by 10^{-6} mol/L 24-eBR or mock treatment for 0.5 h, and then transferred to N-free MS medium for two weeks. After two-week N deprivation, the anthocyanin content was measured in BR related mutants and their corresponding wild-types. (a) Phenotypes of seedlings (Col-0, *bzr1-1D* and *det2*) used in the measurements of anthocyanin content. (b) Anthocyanin levels in Col-0, *bzr1-1D* and *det2*; (c) in *bes1-D* and its wild type En2; (d) and in *bri1-5* and its background Ws.

* Stands for the significant difference between 24-eBR and mock treatment with other conditions the same.

Stands for the significant difference between BR mutants compared to Col-0 under the same treatment.

*/# P value< 0.05; **/## P value< 0.01; ***/### P value< 0.001

3.3 BZR1 interacts with PAP1 *in vitro* and *in vivo*

Previous studies have demonstrated that anthocyanin synthesis in Arabidopsis is regulated by the complexes formed by the WD-repeat protein, the bHLH domain- and the R2R3 MYB domain-containing transcription factors (Hichri *et al.*, 2011). A study done by Lea *et al.* (2006) tested the involvement of different transcription factors in the WD-repeat/bHLH/Myb transcriptional complex in plant N responses, and they found that three factor genes, *PAP1*, *PAP2* and *GL3* showed enhanced expression in response to N deficiency, suggesting that these three factors might be responsible for the regulation of N-deficiency induced anthocyanin synthesis (Lea *et al.*, 2007). Based on this result, we used the yeast two-hybrid assay to investigate possible interactions between three major BR signaling proteins (BIN2, BZR1, BES1) and these three anthocyanin regulators (PAP1, PAP2 and GL3), in an attempt to uncover the underlying mechanisms of BR-enhanced anthocyanin production under N starvation. From the tests, only BZR1 was found to exhibit strong interaction with PAP1 (Fig. 3a), a positive regulator of anthocyanin biosynthesis in Arabidopsis (Borevitz *et al.*, 2000). Although BZR1 and PAP1 both had a close homolog - BES1 and PAP2, respectively, the interaction was exclusively observed between BZR1 and PAP1 (Fig. 3a). This further proved that BZR1, not BES1, mediates the BR regulation of anthocyanin biosynthesis.

We next conducted a bimolecular fluorescence complementation (BiFC) assay to confirm the interaction of BZR1 and PAP1 *in vivo*. BZR1 was fused with the C-terminal fragment of the yellow fluorescence protein (cYFP) to form BZR1-cYFP,

while PAP1 was ligated to the N-terminal fragment of YFP (nYFP) to produce nYFP-PAP1. Both of the constructs were introduced into the leaves of *Nicotiana benthamiana* via *Agrobacterium*-mediated transformation. After 48 hours of incubation, strong YFP fluorescence was observed in the nuclei of epidermal cells in the leaves transformed with the cYFP-BZR1 and nYFP-PAP1 constructs, but not in other construct combinations (Fig. 4c & d). The cellular localization studies of BZR1 and PAP1 using the green fluorescence protein (GFP)–tagged fusion proteins showed that BZR1 expressed in both nucleus and cytoplasm (Fig. 4a) but PAP1 only in nucleus (Fig. 4b).

Since BR can rapidly increase BZR1 level in nucleus (within 0.5 h) (Wang *et al.*, 2002), so it is reasoned that more nucleus-located BZR1 induced by BR treatment will facilitate its interaction with PAP1 to trigger stronger downstream responses.

To further determine the domain of PAP1 required for its interaction with BZR1, N- and C-terminal truncated PAP1 were fused with the GAL4-AD domain of the pGADT7 vector to test their interactions with BZR1 fused with the BD domain in pGBKT7 (Zimmermann *et al.*, 2004). The result showed that the N-terminal R2R3-repeat domain of PAP1 was essential for the interaction with BZR1 (Fig. 3b), but the neither R2- nor R3-repeat domain alone is sufficient to support the interaction with BZR1. We were also intended to test the responsible interacting domain in BZR1 but this was hindered by the strong self-transcription-activation activity when PAP1 was used as the bait.

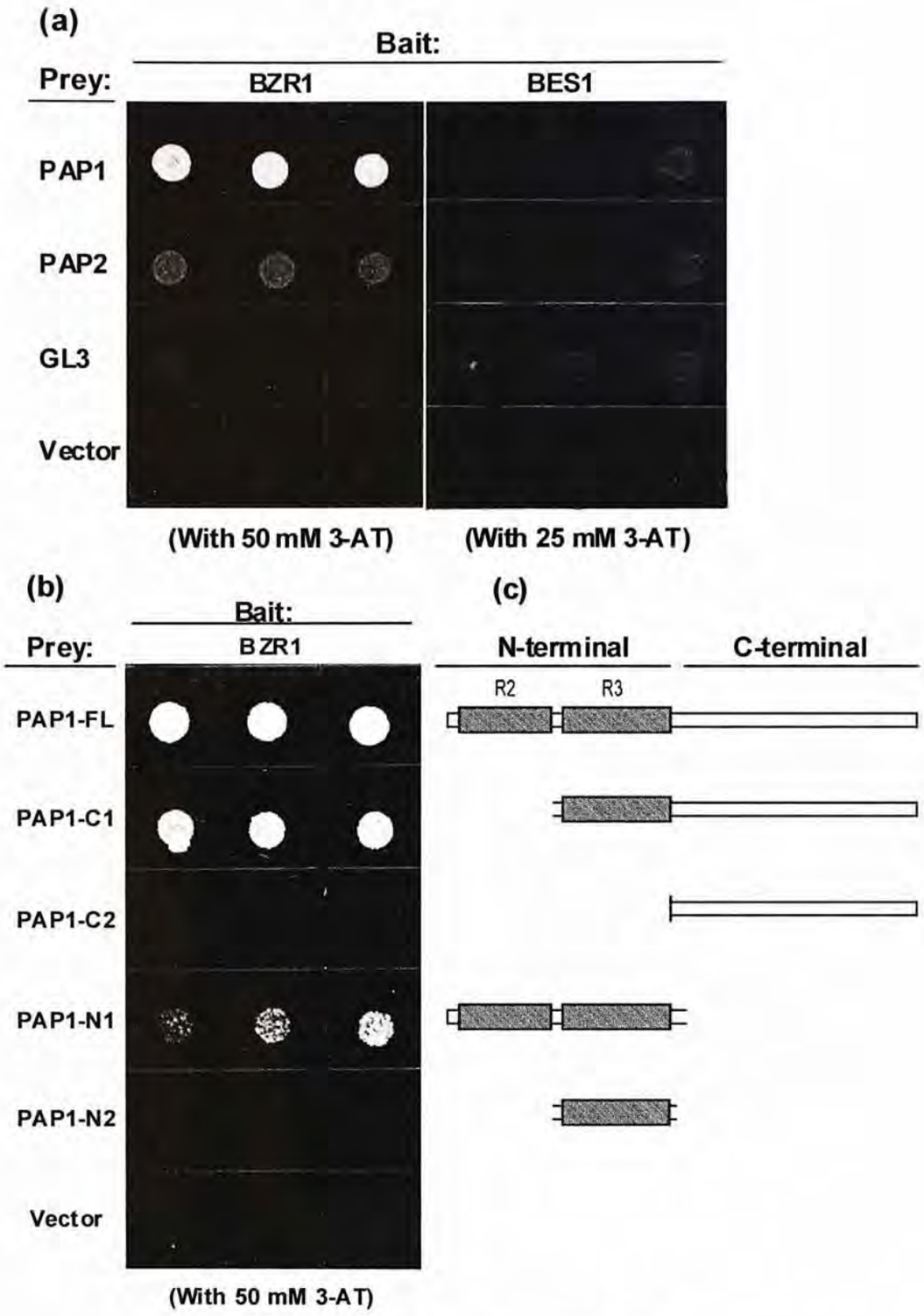


Figure 3. BZR1 and PAP1 interact in yeast

(a) BZR1 and PAP1 interact in yeast. BZR1 or BES1 were fused with the GAL4 DNA-BD domain, while PAP1, PAP2 and GL3 were fused with the AD domain, to investigate the interactions between them. Only BZR1 and PAP1 exhibited positive interaction. (b) The N-terminal domain of PAP1 is responsible for the interaction with BZR1. N- and C-terminal deletion fragments of PAP1 are ligated with the GAL4-AD constructs and used to investigate interactions with BZR1 fused with BD domain. The deletions were shown in (c) PAP1-FL: PAP1 full length; PAP1-C1: R2-repeat domain deletion; PAP1-C2: R2R3-repeat domain deletion (C-terminal fragments); PAP1-N1: R2R3-repeat domain; PAP1-N2: R3-repeat domain; Vector: negative control.

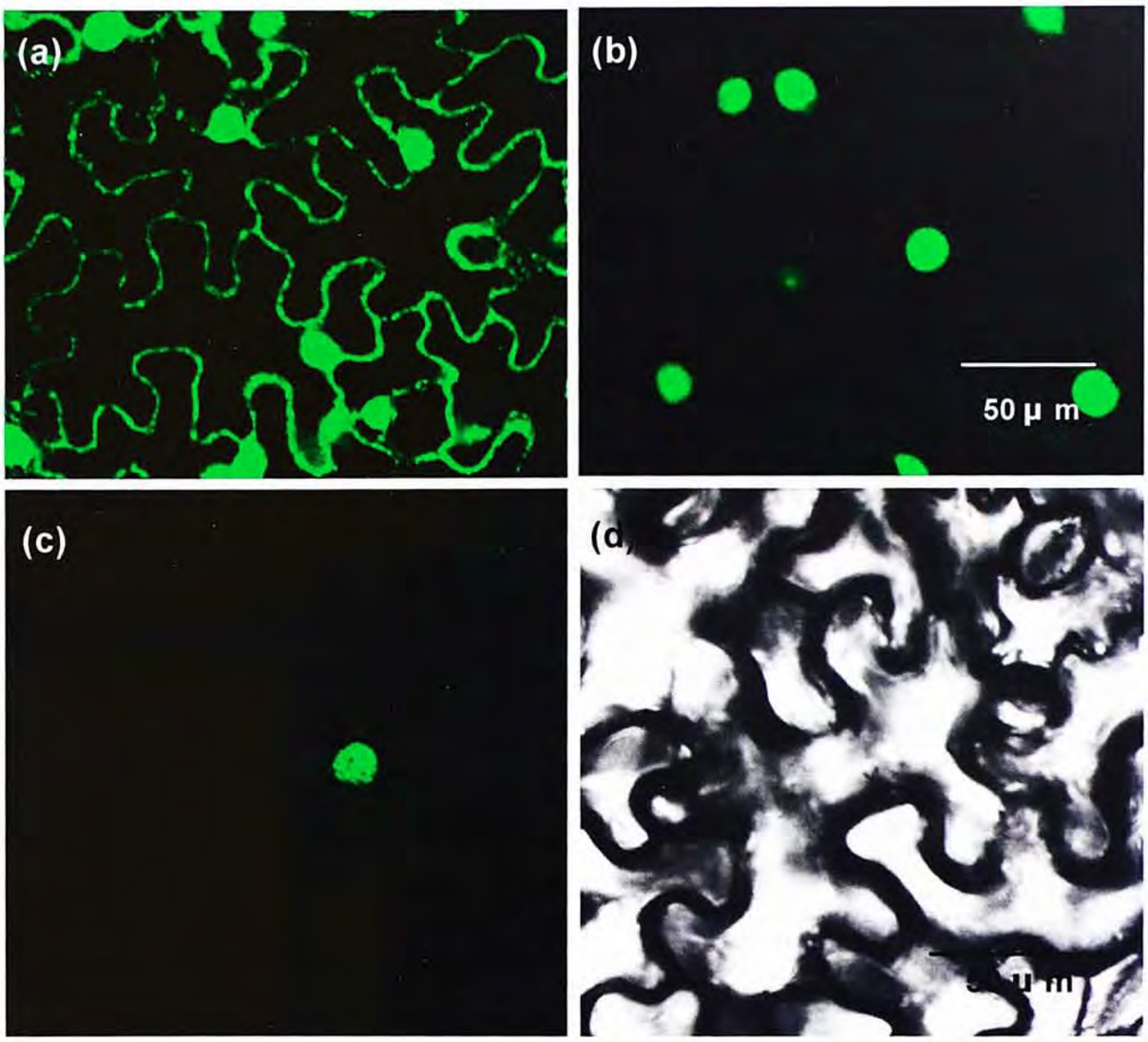


Figure 4. BZR1 and PAP1 interact *in planta*

(a) mBZR1 (with the Pro to Leu mutation) was expressed in both nucleus and cytoplasm. (b) PAP1 was only expressed in nucleus. (c) & (d) Bimolecular fluorescence complementation (BiFC) assays confirmed the interaction of BZR1 protein with PAP1 protein *in vivo*. 48 hours' coexpression of BZR1-cYFP and nYFP- resulted in strong YFP fluorescence in the nucleus of epidermal cells in *N. benthamiana* leaves.

3.4 BR and BZR1 promote the expression of the 'late' anthocyanin biosynthetic genes during N deprivation

Anthocyanins are a group of flavonols and synthesized via a branch of phenylpropanoid pathway. The genes encoding enzymes of every step in the anthocyanin synthetic branch are indispensible for anthocyanin biosynthesis, including the 'early' genes *CHS*, *CHI*, and *F3H*; and the 'late' genes *F3'H*, *DFR*, *ANS*, *AGT*, and *GST* (Winkel-Shirley, 2001; Gonzalez *et al.*, 2008). Moreover, the genes *DFR*, *GST*, *ANS* and *UFGT (UDP flavonoid 5-O-glycosyltransferase)* have been shown to be directly regulated by PAP1 through a conserved PAP1 *cis*-regulatory element in their promoters (Dare *et al.*, 2008).

To understand how BR treatment and *bzr1-1D* mutation lead to enhanced anthocyanin biosynthesis during N starvation, we examined the expression of anthocyanin biosynthetic genes in N-deprived Col-0 and *bzr1-1D* with or without 24-eBR treatment. The expression of all the tested 'early' and 'late' anthocyanin genes (*CHII*, *CHS*, *F3H*, *F3'H*, *DFR*, *ANS*, *AGT*,*UF3GT*, *UFGT* and *GST*), and two genes encoding enzymes on the top of the phenylpropanoid pathway, *PAL* (*phenylalanine ammonia lyase*) and *C4H (cinnamate 4-hydroxylase),* was elevated by N deprivation, but only the 'late' genes *DFR*, *ANS*, *UF3GT*, *UFGT* and *GST* were further enhanced by BR treatment and the *bzr1-1D* mutation (Fig. 5). Interestingly, most of these late genes (*DFR*, *ANS*, *UFGT* and *GST)* are also the direct targets of PAP1. So we infer that the BR-increased anthocyanin accumulation during N starvation was the consequence of promoted expression of the 'late' anthocyanin

synthetic genes *DFR, ANS, UF3GT, UFGT* and *GST.* The consistent responses of the 'late' genes to *bzr1-1D* mutation and PAP1 overexpression, together with the fact that BZR1 interacts with PAP1, strongly suggest that BR-induced anthocyanin biosynthesis is acting though BZR1-PAP1 interaction and their regulation to the 'late' gene expression.

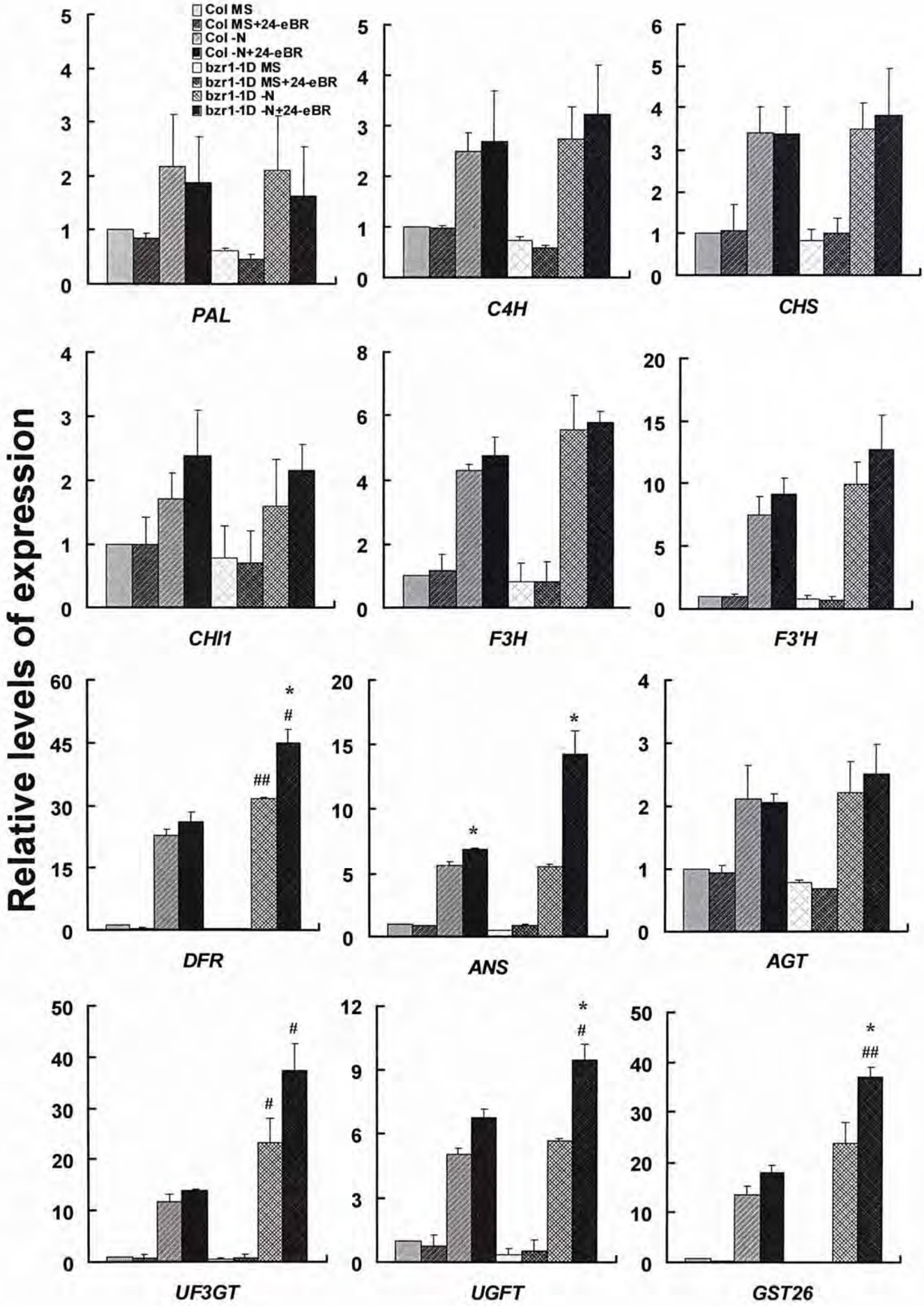

Col MS
Col MS+24-eBR
Col -N
Col -N+24-eBR
bzr1-1D MS
bzr1-1D MS+24-eBR
bzr1-1D -N
bzr1-1D -N+24-eBR
Relative levels of expression
PAL
C4H
CHS
CHI1
F3H
F3'H
DFR
ANS
AGT
UF3GT
UGFT
GST26

Figure 5. The effects of 24-eBR and BZR1 on the expression of anthocyanin synthetic genes

5-day-old Col and *bzr1-1D* seedlings grown on MS medium were treated by 10^{-6} mol/L 24-eBR or mock treatment for 0.5 h, and then transferred to a N-free MS medium for 10 days. The materials were then collected for RNA extraction, reverse transcription and real-time RT PCR analyses. The results showed that BR treatment and BZR1 overexpression only significantly enhanced the expression of specific 'late' genes *DFR, ANS, UF3GT, UFGT* and *GST26,* while have no effect on the 'early' genes.

* Stands for the significant difference between 24-eBR and mock treatment with other conditions the same.

Stands for the significant difference between BR mutants compared to Col-0 under the same treatment.

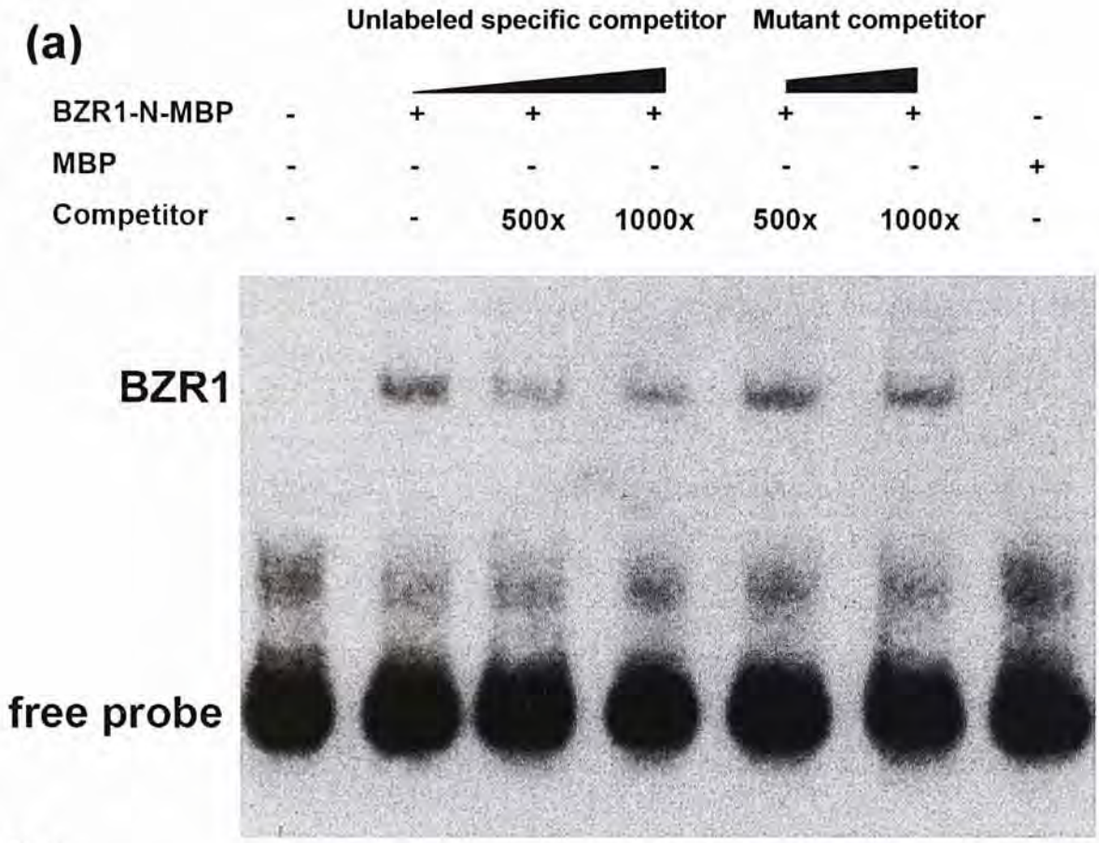
*/# P value< 0.05; **/## P value< 0.01; ***/### P value< 0.001

3.5 BZR1 binds to the promoter of *DFR*

BZR1 as a major transcription factor in BR signaling mediates diverse BR responses either by interacting with other transcription factors or directly binding to the promoters of downstream genes (He *et al.*, 2005, Li and Deng, 2005). Our real-time PCR results showed that some of the 'late' genes (*DFR, ANS, UF3GT, UFGT* and *GST*) in anthocyanin biosynthetic pathway are responsive to both *bzr1-1D* mutation and BR treatment. This might be an indirect effect of BZR1 through its interaction with PAP1, as most of the genes are direct targets of PAP1 (Dare *et al.*, 2008), or it's possible that these genes are also directly regulated by BZR1. To determine whether BZR1 regulates these 'late' genes (*DFR, ANS, UF3GT, UFGT* and *GST*), EMSAs were conducted to study the potential binding ability of BZR1 protein to the promoters of these 'late' genes. Among these genes, only *DFR* and *F3'H* have a highly conserved BRRE in their promoters. However, only the *DFR* promoter can bind to the BZR1 protein. It is intriguing that both *DFR* and *F3'H* gene promoters contain the same conserved BRRE sequence, but only *DFR*, not *F3'H* promoter could bind BZR1. This is in agreement with results from gene expression analyses, which also showed that only the expression of *DFR* is regulated by BZR1 but not that of *F3'H* (Fig. 5). This result also suggests that other sequences flanking BRRE are also important for specific binding between BZR1 and its target DNA sequences. While the competition assay confirmed the specificity of the binding between BZR1 and *DFR* promoter, the competitor containing BRRE-mutation (AAAAAA) has no effect on the DNA-protein binding, indicating that BRRE is the

binding site mediating BZR1-*DFR* interaction (Fig. 6a).

However, since *DFR* is also a direct target of PAP1 protein as well as the PAP1/bHLH complex, with respect to the mechanisms of the BZR1-PAP1 interaction in controlling *DFR* expression, it's possible that BZR1 enhances the PAP1-DFR interaction through its binding at a nearby site in *DFR* promoter, leading to promoted *DFR* expression (Fig. 6b).



(b)

atccacgtggacgaggtaa**ccaccacgtg**ttatttcttactttatgagattaagtgattcactgtccttctaattttatttatttttctttgcgaaa

PAP1 binding site 1

tctggaaataaataaatttttgaaaacaatattttaacatatcaattatttttataataattcgtgaaataataatgaaaaagctgacatgggacacaatt
ggataattacatcttttaaagttttagtttatgtatttttatgtaaatgcttataatatttagattgcaataaatctagaagtcatatttataaattaatatgttttata
attttacaattttgtaataagaaactcctaattcataaatctaaaatataaacatatttttcatttaagcttttccaagatttataattattttaggtgtctgatttt
agatttcaattaaaattaaaatattacttaagtaaaatgtatttctgtatatattctatcaaaatgttaatttgtttagacaaattttgatttatttcgtaaaagtg
ggtggggaacaaaaacaaaaacaaactgaactgaagtcacc**cacacg**tctcaccaaacaaatcgaagtcaacgtatttcaccca

BZR1 binding site

ccggtacaacaacaaaatacacacctaaggaaataataaaatcaacttaccagattgttacgtaccacacatctctttagtccttcgtcaaccaac
gt**tccccacgtg**cttctccggttggtactcacgtgaccggcagcttctcgttcttattatctgttttcttcaataacgattcataatctctagtgtctt

PAP1 binding site 2

atttataatgtcttcacatcacaaagatttgtaccgaacatacatagttgaatctttcccaaagcacaatctatcatataaccacaaaa**ATG**

Figure 6. Electrophoretic mobility shift assay (EMSA) for interaction of BZR1 and *DFR* promoter

(a) BZR1 binds to the probe containing a BRRE (cacacg) sequence from the *DFR* promoter. The upper bands were the probes bound with BZR1, which can be reduced by unlabeled native competitors but not by the mutant competitor. (b) Promoter sequence of the *DFR* gene which contains a highly conserved BRRE (cacacg) and two PAP1 binding sites as indicated.

3.6 BR-enhanced anthocyanin accumulation is specific to N-deprivation

As the primary nutrients of plants, N, Pi and K are all important for plant growth and development. Deficiency of any of them will cause profound morphological and metabolic responses (Peng *et al.*, 2008a; Jiang *et al.*, 2007). In plants, the visible accumulation of anthocyanin is one of the characteristic responses to N and Pi starvation (Lillo *et al.*, 2008). However, we found that the Pi-starvation induced anthocyain accumulaiton was not enhanced by 24-eBR or *bzr1-1D* mutation (Fig. 7). In fact, in *bzr1-1D* seedlings, the anthocyanin content was even lowered by BR treatment. The same experiment was carried out for K starvation. However, compared to the N and Pi starvation, the deprivation of K under our experimental conditions didn't induce noticeable anthocyanin accumulation, which was not affected by BR treatment or *bzr1-1D* mutation either (Fig. 7). Altogether, our results suggest that even both N and Pi limitation can induce anthocyanin biosynthesis but the BR-enhanced anthocyanin accumulation was low N-specific.

It was supposed that various combinations of different regulators in the WD-repeat/bHLH/Myb complex may specifically regulate the structural genes and response to different kinds of stimuli (Nesi *et al.*, 2001; Quattrocchio *et al.*, 1998). A best example comes from the finding that three MYB-related transcription factors regulate the anthocyanin structural genes with different specificities, which results in different patterns and intensity of anthocyanin pigmentation in *Antirrhinum* flowers (Schwinn and Venail, 2006).

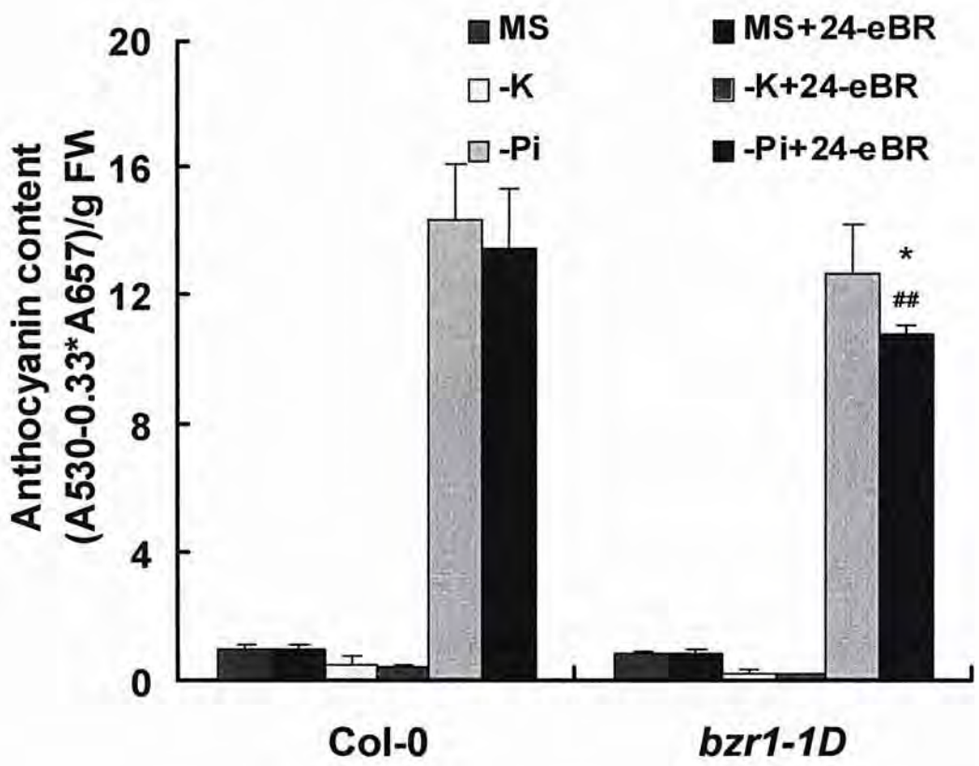


Figure 7. BR does not enhance anthocyanin accumulation under other nutrient deprivation conditions

5-day-old WT (Col-0) and *bzr1-1D* seedlings grown on MS medium were treated by 10^{-6} mol/L 24-eBR or mock treatment for 0.5 h, transferred to Pi and K deprivation media for two weeks and then the anthocyanin content was measured.

* Stands for the significant difference between 24-eBR and mock treatment with other conditions the same.

Stands for the significant difference between BR mutants compared to Col-0 under the same treatment.

*/# P value< 0.05; **/## P value< 0.01; ***/### P value< 0.001

3.7 BZR1 differently regulates *PAP1* and *PAP2*

Plants have evolved various regulators to control the anthocyanin biosynthesis in the form of a WD-repeat/bHLH/Myb complex, such as TTG1, PAP1, PAP2, GL3, and EGL3 *etc*. Various combinations of different regulators in the WD-repeat/bHLH/Myb complex may respond to different kinds of stimuli and specifically regulate the structural genes (Quattrocchio *et al*., 1998; Nesi *et al*., 2001). Our data have demonstrated that BZR1 interacts with PAP1 and enhances the expressions of PAP1-downstream genes ('late' anthocyanin structure genes), which indicates a positive regulation of BZR1 on PAP1. However, our real-time PCR results showed that BZR1 did not significantly regulate the expression level of *PAP1*, but interestingly, down regulated the expression of *PAP2*, a close homolog of *PAP1*, (Fig. 8a). Furthermore, we found that there are 4 BRREs (tacacg) in the *PAP1* promoter, and 7 BRREs (including 4 tacacg and 3 cgtgca) in the *PAP2* promoter. Thus, EMSAs were conducted to study the potential binding between BZR1 protein and the *PAP1/PAP2* promoters. Consistent with the real-time PCR results, we found that only the promoter of *PAP2* not that of *PAP1* could bind to the BZR1 protein (Fig. 8b). This result suggests again that the BRRE-flanking sequences are also important for specific binding between BZR1 and its target DNA sequences. The competition assays using the unlabeled *PAP2* promoters with or without the BRRE-mutated sequences as competitors confirmed that BRRE is the binding site mediating BZR1-*PAP2* interaction (Fig. 8b).

So, BZR1 positively regulates PAP1 through protein-protein interaction, whereas

it negatively regulates PAP2 through protein-DNA interaction. This interesting result might represent part of the control mechanisms for anthocyanin biosynthesis in plant response to different stimuli. It has been demonstrated by previous studies that, compared to PAP1, the expression level of *PAP2* is extremely low under non-stressful conditions (Lea *et al.*, 2007). That means the transcription of *PAP2* is actually inhibited under normal growth conditions. There is a possibility that this inhibition is mediated by BZR1. However, it's hard to be confirmed due to the extreme low expression level of *PAP2* under non-stressful conditions.

Studies have shown that N starvation could dramatically increase the expression level of *PAP2.* For example, a 200-times-higher expression of PAP2 over PAP1was observed by Lea *et al.* (2007). Based on this finding, previous study had suggested that *PAP2* may play a bigger role in the N-deprivation-induced anthocyanin accumulation (Lea *et al.* 2007). In our study, the Low-N-induced *PAP2* expression was more than 300 times higher than that of *PAP1*. However, our anthocyanin measurements in N-depleted *pap2* mutant (a T-DNA insertion line) (Alonso et al., 2003) indicated that the *pap2* mutant has similar anthocyanin contents to the wild-type Col-0 (Fig. 9a & c). Meanwhile, the BR-increased anthocyanins were not affected in the mutant either (Fig. 9a & c). Thus, we reasoned that, although the expression level of PAP2 is strongly promoted by N starvation, it's still not the major regulator of the N-deprivation-induced anthocyanin biosynthesis. This also explained why the dramatically inhibited *PAP2* expression did not cause decrease in anthocyanin contents in the *bzr1-1D* mutant.

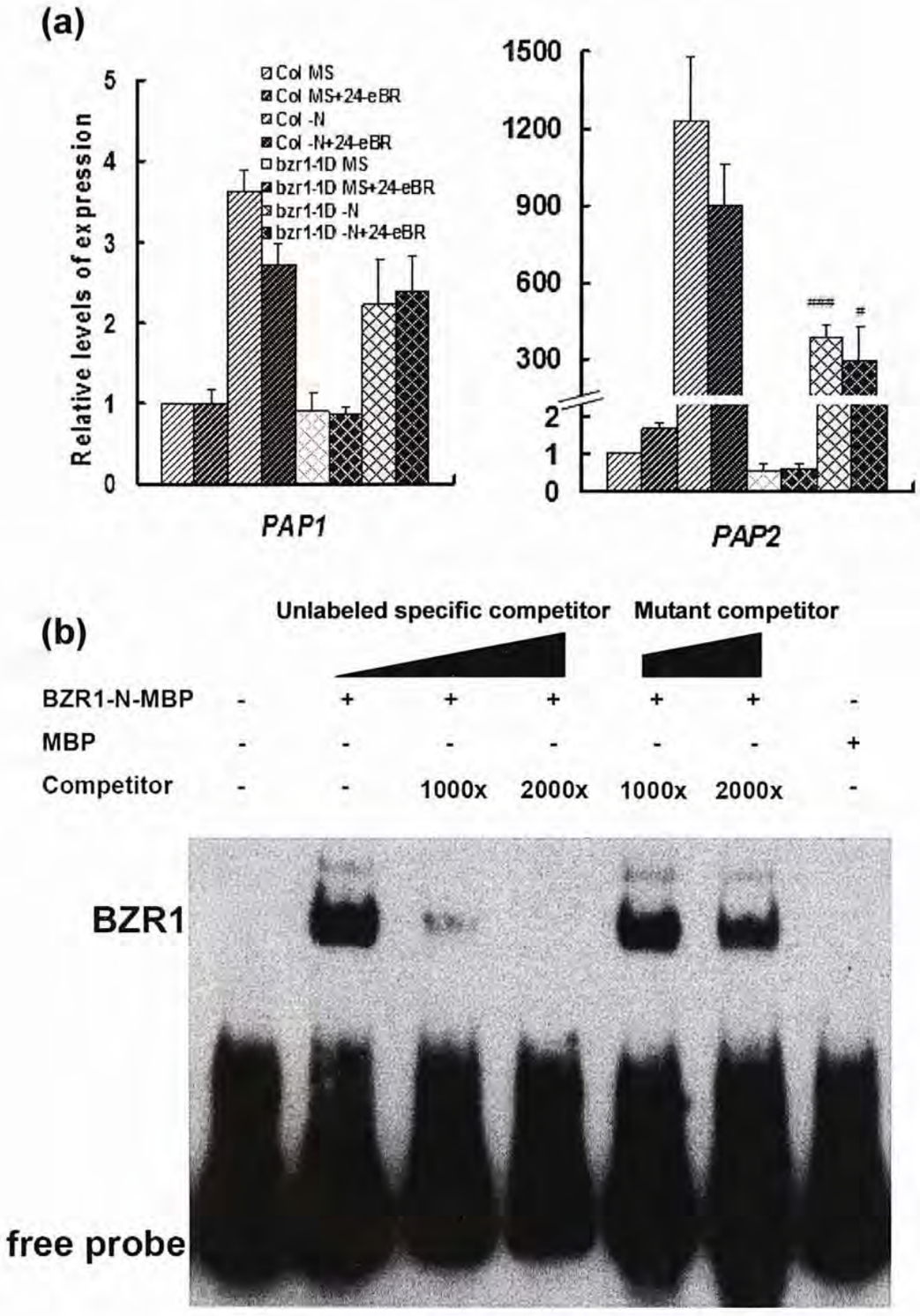


Figure 8. BZR1 differently regulates *PAP1* and *PAP2* expression

(a) 5-day-old Col-0 and *bzr1-1D* seedlings grown on MS medium were treated by 10-6 mol/L 24-eBR or mock treatment for 0.5 h, and then transferred to a N-free MS medium for 10 days. The materials were then collected for RNA extraction, reverse transcription and real-time RT PCR analyses. The results showed that BR treatment and *bzr1-1D* mutation only significantly affected the expression of *PAP2* but not *PAP1* (b) BZR1 binds to the probe containing BRREs from the *PAP2* promoter. The upper bands were the probes bound with BZR1, which can be reduced by unlabeled native competitors but not by the mutant competitor.

* Stands for the significant difference between 24-eBR and mock treatment with other conditions the same.

Stands for the significant difference between BR mutants compared to Col-0 under the same treatment.

*/# P value< 0.05; **/## P value< 0.01; ***/### P value< 0.001

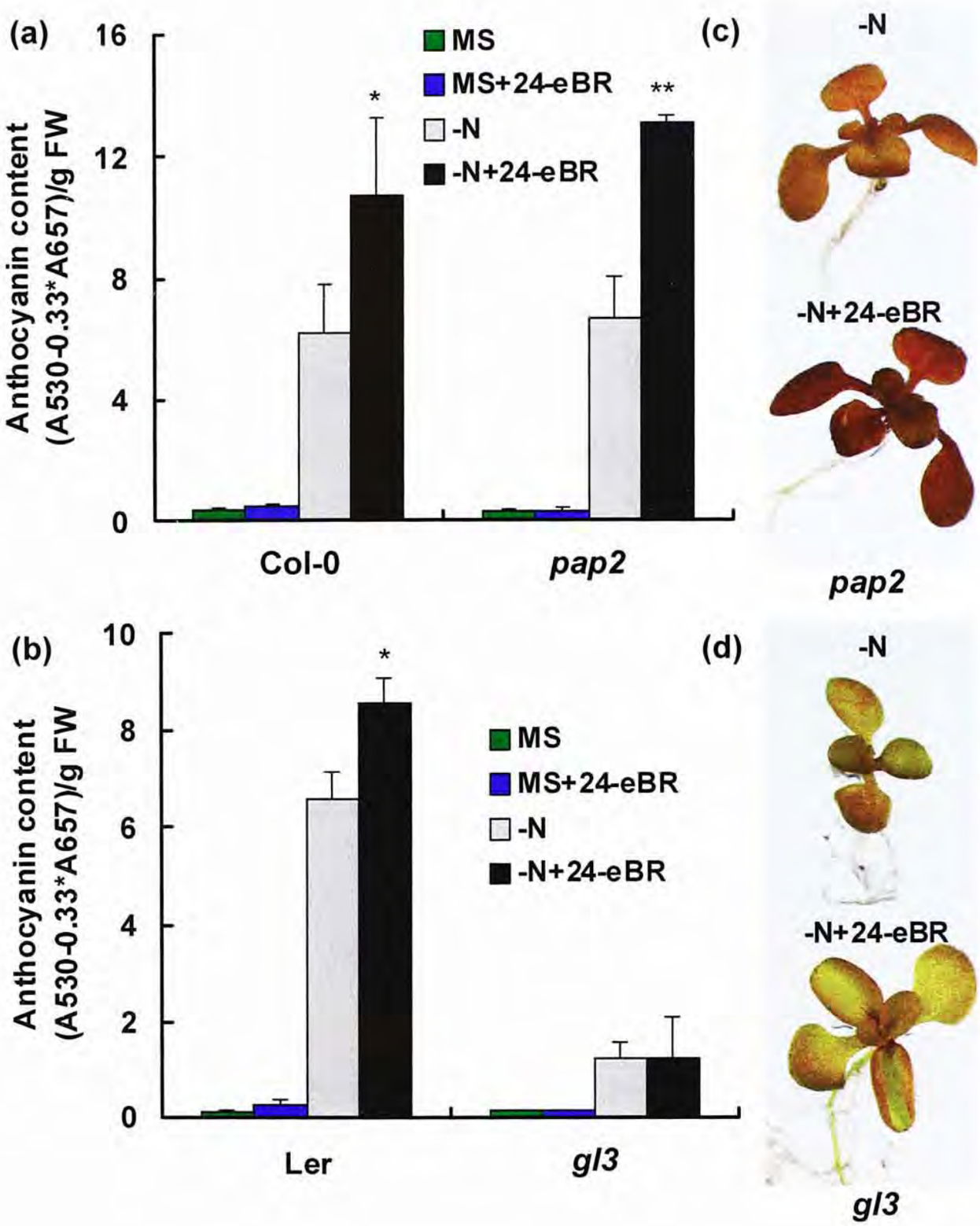


Figure 9. Anthocyanin accumulation in *pap2* and *gl3* under N deprivation

5-day-old seedlings grown on MS medium were treated by 10^{-6} mol/L 24-eBR or mock treatment for 0.5 h, and then transferred to N-free MS medium for two weeks. After two-week N deprivation, the anthocyanin content was measured in *pap2* and *gl3* mutants and their corresponding wild-types. (a) Anthocyanin contents in Col-0 and *pap2*; (b) in Ler and *gl3*. (c) Phenotypes of N-deprived *pap2* and (d) *gl3* with or without 24-eBR treatment.

3.8 Endogenous *GL3* is required for BR-enhanced anthocyanin biosynthesis

Previous study has shown that endogenous *GL3* is not replaced by *EGL3* or other bHLH transcription factors in Arabidopsis leaves to induce anthocyanins under N deprivation stress (Feyissa *et al.*, 2009). N-deprivation induced anthocyanins were absent in cotyledons and rosette leaves of the *gl3* mutant, while the wild-type and *egl3* mutant both normally accumulated anthocyanins in response to N depletion (Feyissa *et al.*, 2009). Here we found that endogenous *GL3* is also required in the BR-enhanced anthocyanin synthesis, as the absent anthocyanins in *gl3* mutant were not recovered by BR treatment (Fig. 9b & d). That means the *GL3* is downstream of BR signaling in the regulation of anthocyanin biosyntheisis. It's possible that GL3 is the bHLH factor together with PAP1 and TTG1 to form the regulatory complex in response to BR-regulated anthocyanin biosynthesis under N starvation.

3.9 N status affects the expression of BR biosynthetic gene *CPD*

Previous studies have shown that nitrate affected the expression of CK biosynthetic genes *AtIPT3* and *AtIPT5* (Franco-Zorrilla *et al.*, 2005), suggesting an interaction between CK and N signaling. Here we found that the expression of *CPD,* a BR biosynthetic gene, was also regulated by nitrate. Real-time PCR analyses showed that the expression of *CPD* is down-regulated by BR treatment, *bzr1-1D* mutation, as well as N starvation (Fig. 10a). However, the results of RT-PCR (Fig. 10b) and GUS staining (Fig. 10c) showed a dynamic response of this gene to different nitrate levels: low nitrate concentration inhibits its expression, while high

concentration of nitrate enhances its expression, suggesting the important role of N status in BR biosynthesis.

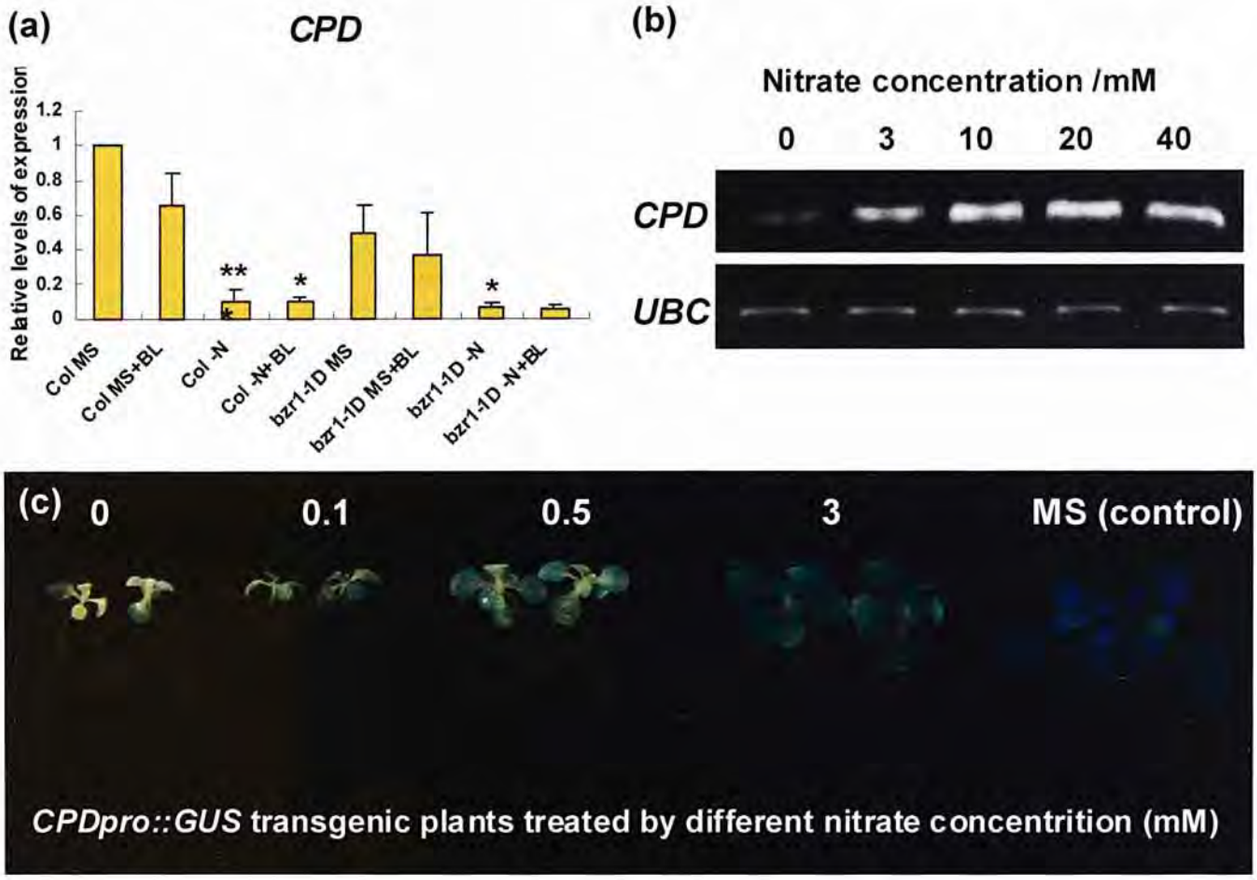


Figure 10. The expression of the BR biosynthetic *CPD* gene is affected by plant N status

(a) Nitrogen starvation suppresses the expression of *CPD;* (b) Nitrate concentrations affect the expression of *CPD;* (c) *CPD::GUS* transgenic plants treated with different nitrate concentrations.

* Stands for the significant difference between N deprivation treatment and mock treatment.

Part 4 Discussion

4.1 BRs confer plant tolerance to low-N stress and the tolerance is mediated by BR enhancement of low-N-induced anthocyanin biosynthesis

In addition to their essential roles in plant growth, studies have shown that BRs also play important roles in plant tolerance to different stresses including heat, cold, drought, salinity and pathogen attack (Krishna, 2003). For example, 24-eBR was shown to increase the basic thermotolerance in several plant species by protecting the translational machinery and accumulating higher levels of major heat shock proteins under thermal stress (Dhaubhadel *et al.*, 1999; Dhaubhadel *et al.*, 2002). BR also enhances drought tolerance of Arabidopsis and *Brassica napus*, as 24-eBR treatment can increase the recovery rate of the plants under drought stress by 2 to 4-fold (Dhaubhadel *et al.*, 1999). However, whether BRs or BR signaling are involved in nutrient stresses in plants is still unknown. Here, we found that exogenous BR treatment can protect Arabidopsis seedlings from N starvation stress which was, at least in part, mediated by the transcription factor BZR1. While about 70% of wild-type Arabidopsis seedlings (Col-0) cannot survive 40-d's N deprivation, more than 90% of 24-eBR-treated Col-0 and the untreated *bzr1-1D* mutants were recoverable after transferring back to the +N medium (Fig. 1). Since both BR treatment and *bzr1–1D* mutation increase BZR1 protein accumulation and activity (Wang *et al.*, 2002), our results suggest that the BZR1 transcription factor plays an important role in BR-mediated plant tolerance to N starvation.

Although it has been well known that BRs can increase plant tolerance to a variety of environmental stresses, the underlying molecular mechanisms are poorly understood. Previous studies have demonstrated that anthocyanin accumulation is important for Arabidopsis adaptation to N limitation (Peng *et al.*, 2008a). In this study, we found that both BR treatment and the *bzr1-1D* mutant can increase plant tolerance to N-deprivation stress, and the low N-induced anthocyanin accumulation was enhanced in BR-treated wild-type plants and in the *bzr1-1D* mutants. These results suggest a clear correlation between BR-induced plant tolerance and its enhancement of anthocyanin biosynthesis under low N stress.

4.2 BRs enhance anthocyanin accumulation under N starvation through BZR1-PAP1 interaction or direct control of the expression of anthocyanin biosynthetic genes

To understand how BRs enhance low-N-induced anthocyanin biosynthesis and thereby increasing plant tolerance to low N stress, we tested possible interactions between key BR signaling components (BIN2, BZR1 and BES1) and three transcription factors (PAP1, PAP2 and GL3) that are known to control low N-induced anthocyanin accumulation. From these tests, we detected strong interaction between BZR1 and PAP1, and weak interaction between BZR1 and PAP2. It is interesting that although BZR1 and BES1 are close homologs, we did not observe an interaction between BES1 and PAP1 (Fig. 3a). Therefore, our results in this study present a novel evidence for distinct functions of BZR1 and BES1 in BR signaling and plant

development. In line with this assumption, the *bes1-D* mutant which has the same point mutation with *bzr1-1D*, did not show increased anthocyanin accumulation under N starvation condition (Fig. 2c), suggesting that only BZR1 not BES1 is involved in the BR-enhanced anthocyanin biosynthesis. The finding of physical interaction between BZR1 and PAP1 (a positive regulator of anthocyanin synthesis) confirmed the involvement of BR signaling, particularly BZR1, in anthocyanin accumulation under N deprivation. BZR1 may promote the expression of certain 'late' anthocyanin structure genes through its interaction with PAP1 under N deprivation, as most of the structure genes (*DFR, UFGT* and *GST*) are also the targets of the PAP1 transcription factor (Fig. 5). Furthermore, PAP1 and BZR1 both can bind to the promoter of *DFR* gene through nearby binding sites, suggesting that in addition to interacting with PAP1, BZR1 may also enhance anthocyanin biosynthesis by directly controlling the expression of the *DFR* gene (Fig. 6).

4.3 BRs are specifically involved in low-N induced anthocyanin production

It is noteworthy that under normal N conditions, can neither BR treatment nor *bzr1-1D* mutation induce visible anthocyanin accumulation in Arabidopsis, possibly due to the rather low level of PAP1 expression. However, this notion was not supported by the results from Pi starvation experiments, where although the expression level of PAP1 was greatly increased (Jiang *et al.*, 2007), we did not see a BR-enhancement of anthocyanin accumulation in both Col-0 and *bzr1-1D* (Fig. 7). This means that the BR-promoted anthocyanin synthesis is a rather specific process

to N deprivation. The study of the *nla* mutant in Arabidopsis has suggested that plants have evolved a specific pathway to regulate anthocyanin biosynthesis under N limitation, which is supported by the facts that the *nla* mutation disrupts N-limitation induced anthocyanin synthesis but does not affect the Pi-limitation induced anthocyanin synthesis (Peng *et al.*, 2008a). So in Arabidopsis, it's highly possible that BZR1 mediates a specific signaling pathway to control the N-deprivation induced anthocyanin production through its interaction with PAP1.

4.4 Transcription factors that specifically control BR-regulated anthocyanin biosynthesis

Previous studies have demonstrated that anthocyanin synthesis in Arabidopsis is regulated by the WD-repeat/bHLH/Myb complexes (Hichri *et al.*, 2011). TTG1 as a conserved regulator within plant kingdom plays a central role in the production of anthocyanin and other secondary metabolism processes. Studies have indicated that TTG1 is constitutively expressed; its abundance *in planta* is not influenced by nutrient deprivation in Arabidopsis (Lillo *et al.*, 2008). Various combinations of MYB and bHLH factors in the WD-repeat/bHLH/Myb complex may specifically regulate anthocyanin synthesis in response to different kinds of stimuli (Quattrocchio *et al.*, 1998; Nesi *et al.*, 2001) A study by Lea *et al.* (2007) found that three transcription factors, *PAP1*, *PAP2* and *GL3* showed enhanced expression in response to N deficiency, suggesting important roles of these three factors in the regulation of low N-induced anthocyanin synthesis (Lea *et al.*, 2007).

Under N deprivation, the absence of anthocyanins in cotyledons and rosette leaves of the *gl3* mutant suggests critical role of endogenous *GL3* in the N-deprivation-induced anthocyanin biosynthesis (Feyissa *et al.*, 2009). In our experiments, we also found that *GL3* is required for BR-enhanced anthocyanin production, as the *gl3* not *egl3* mutant (data not shown) is able to destruct the BR-enhanced anthocyanin content under N deprivation (Fig. 9b). Although the expression level of *PAP2* was strongly induced by N starvation (by more than 1000-fold change) (Fig. 8a), our experiment with the *pap2* mutant indicated that the anthocyanin level was not affected by the *pap2* mutation under N deprivation (Fig. 9a), suggesting that PAP2 is not the major regulator of low N-induced anthocyanin biosynthesis. As discussed earlier, BR enhancement of anthocyanin accumulation under N starvation is through BZR1-PAP1 interaction. Therefore, PAP1 plays a major role not only in low N-induced anthocyanin biosynthesis but also in BR-mediated enhancement of anthocyanin biosynthesis under low N stress. Taken together, the results from this study established that two factors PAP1 and GL3 are specifically involved in BR-mediated anthocyanin biosynthesis in Arabidopsis, with PAP1 playing a major role.

4.5 *DFR* is an important target of BR regulation of anthocyanin biosynthesis

To further understand the molecular mechanism of BR regulation of anthocyanin biosynthesis, we tested by EMSA assays whether BZR1 can bind the promoters of any of the anthocyanin biosynthetic genes to directly control their gene expression.

In agreement with our finding that BZR1 binding sites were present in the promoters of some of the anthocyanin structure genes including *DFR*, strong interaction was observed between BZR1 and the *DFR* promoter but not other promoters (Fig. 6), suggesting that *DFR* is a direct target of BZR1 and it plays a crucial role in BR-regulated anthocyanin biosynthesis. This notion was further supported by the results from quantitative RT-PCR analyses which showed that *DFR* expression is strongly induced by the *bzr1-1D* mutation (Fig. 5).

Part 5: Conclusions

In this study we demonstrate for the first time that BRs can increase the tolerance of Arabidopsis plants to the N starvation stress through enhancing anthocyanin accumulation under N starvation. Mechanistically, we found that BZR1 interacts with PAP1, and increases the expression of several 'late' anthocyanin biosynthetic genes under N starvation conditions. BZR1 was also found to specifically bind the promoter of *DFR*, a key "late" gene for anthocyanin biosynthesis, suggesting that BR can directly control the expression of anthocuanin biosynthetic genes (Fig. 11). Finally, we found that the BR-enhancement of anthocyanin accumulation was specific to plant response to low N stress, as neither 24-eBR treatment nor *bzr1-1D* mutation had a positive effect on the low-Pi-induced anthocyanin biosynthesis in Arabidopsis plants. Above all, these results advanced our knowledge of BR functions in plant growth and development and more importantly, provided new insights into the molecular mechanisms by which BRs regulate stress tolerance in plants.

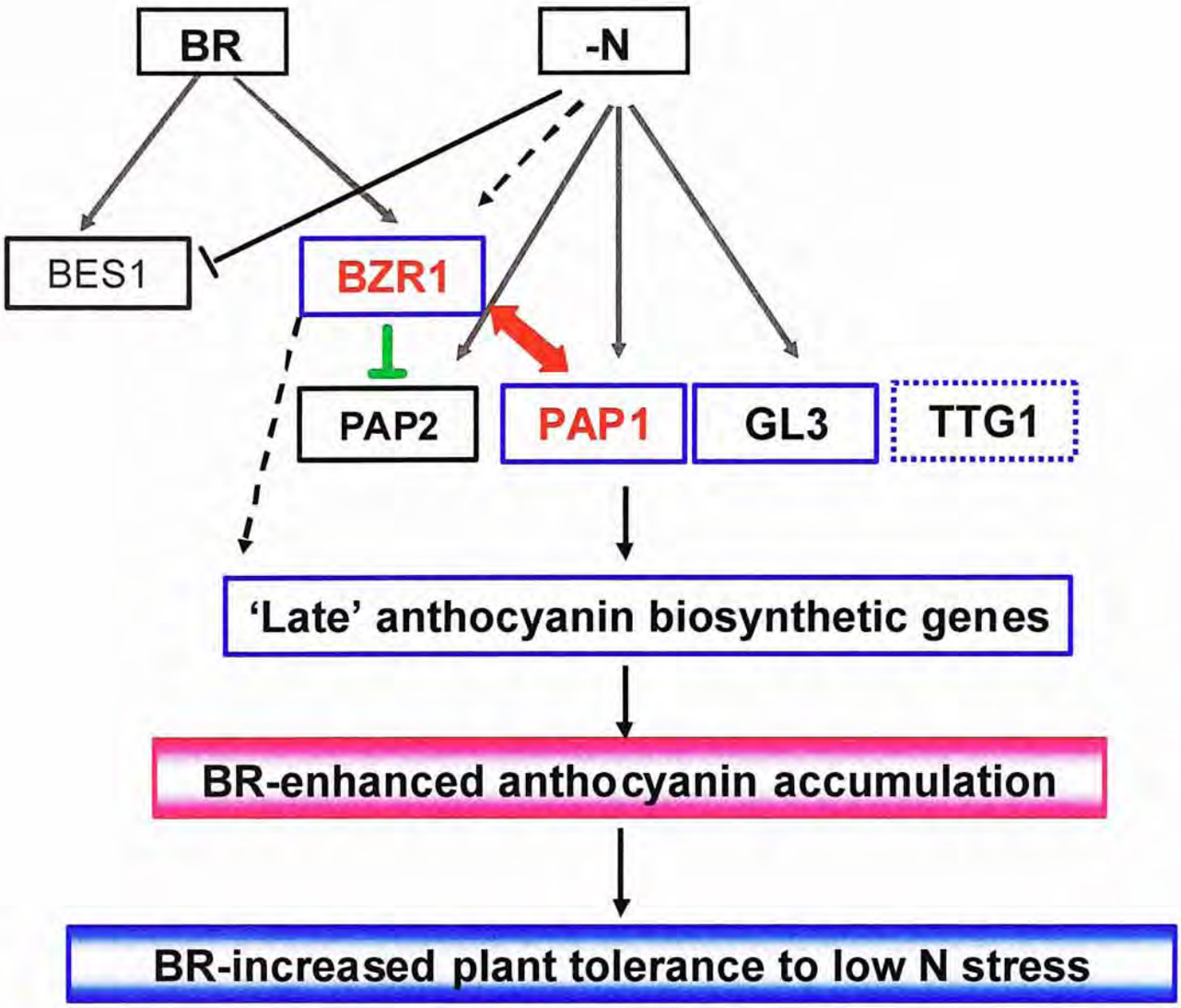


Figure 11. Working model of BZR1 mediated anthocyanin accumulation and plant tolerance under N deprivation stress

BR promotes the accumulation and activity of both BZR1 and BES1. It has been shown that N deprivation induces the expression of *PAP1*, *PAP2* and *GL3* (the grey arrows). In this study, we find that N deprivation can slightly increase the expression of *BZR1* while significantly decrease the expression of *BES1*, indicting an important role of *BZR1* not *BES1* in BR signaling to respond to low-N stress. Further studies have shown that BZR1 dramatically inhibits the N-deprivation-induced *PAP2* expression by directly binding to the promoter of *PAP2*, while positively regulates PAP1 through protein-protein interaction. Under N deprivation, the BZR1-PAP1 interaction may mediate the promoted expression of 'late' anthocyanin biosynthetic genes, which further leads to the BR-enhanced anthocyanin accumulation and results in the BR-increased plant tolerance to low-N stress. At the same time, BZR1 also directly binds to the promoter of 'late' gene - *DFR*. The BZR1-*DFR* interaction may also promote the expression of *DFR* under N deprivation stress.

Part 6: References

ACEBEDO, S. L., ALONSO, F., GALAGOVSKY, L. R. & RAMIREZ, J. A. 2011. Synthesis and biological activity of ring-A difluorinated brassinosteroids. *Steroids*.

AKERSTROM, A., FORSUM, A., RUMPUNEN, K., JADERLUND, A. & BANG, U. 2009. Effects of sampling time and nitrogen fertilization on anthocyanidin levels in Vaccinium myrtillus fruits. *J Agric Food Chem,* 57, 3340-5.

ALONSO, J., STEPANOVA, A., & LEISSE, T. *et. al.* 2003. Genome-wide insertional mutagenesis of Arabidopsis thaliana. *Science,* 301, 653-657

ALONSO-RAMIREZ, A., RODRIGUEZ, D., REYES, D., JIMENEZ, J. A., NICOLAS, G., LOPEZ-CLIMENT, M., GOMEZ-CADENAS, A. & NICOLAS, C. 2009. Cross-talk between gibberellins and salicylic acid in early stress responses in Arabidopsis thaliana seeds. *Plant Signal Behav,* 4, 750-1.

ALTMANN, T. 1998. Recent advances in brassinosteroid molecular genetics. *Curr Opin Plant Biol,* 1, 378-83.

AN, C. & MOU, Z. 2011. Salicylic Acid and its function in plant immunity(f). *J Integr Plant Biol,* 53, 412-28.

ANTONCHICK, A. P., SCHNEIDER, B., ZHABINSKII, V. N. & KHRIPACH, V. A. 2004. Synthesis of [26,27-2H6]brassinosteroids from 23,24-bisnorcholenic acid methyl ester. *Steroids,* 69, 617-28.

ARNON, D. I. & STOUT, P. R. 1939. The Essentiality of Certain Elements in Minute Quantity for Plants with Special Reference to Copper. *Plant Physiol,* 14, 371-5.

ASAMI, T., MIN, Y. K., NAGATA, N., YAMAGISHI, K., TAKATSUTO, S., FUJIOKA, S., MUROFUSHI, N., YAMAGUCHI, I. & YOSHIDA, S. 2000. Characterization of brassinazole, a triazole-type brassinosteroid biosynthesis inhibitor. *Plant Physiol,* 123, 93-100.

ASAMI, T. & YOSHIDA, S. 1999. Brassinosteroid biosynthesis inhibitors. *Trends Plant Sci,* 4, 348-353.

AZPIROZ, R., WU, Y., LOCASCIO, J. C. & FELDMANN, K. A. 1998. An Arabidopsis brassinosteroid-dependent mutant is blocked in cell elongation. *Plant Cell,* 10, 219-30.

BAJGUZ, A. & HAYAT, S. 2009. Effect of brassinosteroids on the plant responses to environmental stresses. *Plant Physiology and Biochemistry*, 47: 1-8.

BERLINER, M. D. 1981. Hormone effects on Cosmarium botrytis cell division. *Cytobios,* 30, 89-99.

BEZRUKOVA, M. V., AVAL'BAEV, A. M., KIL'DIBEKOVA, A. R., FATKHUTDINOVA, R. A. & SHAKIROVA, F. M. 2002. Interaction of wheat lectin with 24-epibrassinolide in the regulation of cell division in wheat roots. *Dokl Biol Sci,* 387, 533-5.

BHAT, R. A., LAHAYE, T. & PANSTRUGA, R. 2006. The visible touch: in planta visualization of protein-protein interactions by fluorophore-based methods. *Plant Methods,* 2, 12.

BISHOP, G. J. & YOKOTA, T. 2001. Plants steroid hormones, brassinosteroids: current highlights of molecular aspects on their synthesis/metabolism, transport, perception and response. *Plant Cell Physiol,* 42, 114-20.

BLAZQUEZ, M. A., GREEN, R., NILSSON, O., SUSSMAN, M. R. & WEIGEL, D. 1998. Gibberellins promote flowering of arabidopsis by activating the LEAFY promoter. *Plant Cell,* 10, 791-800.

BLOOR, S. J. & ABRAHAMS, S. 2002. The structure of the major anthocyanin in Arabidopsis thaliana. *Phytochemistry,* 59, 343-6.

BONHOMME, F., KURZ, B., MELZER, S., BERNIER, G. & JACQMARD, A. 2000. Cytokinin and gibberellin activate SaMADS A, a gene apparently involved in regulation of the floral transition in Sinapis alba. *Plant J,* 24, 103-11.

BOREVITZ, J. O., XIA, Y., BLOUNT, J., DIXON, R. A. & LAMB, C. 2000. Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. *Plant Cell,* 12, 2383-2394.

BOUKCIM, H., PAGES, L. & MOUSAIN, D. 2006. Local NO3- or NH4+ supply modifies the root system architecture of Cedrus atlantica seedlings grown in a split-root device. *J Plant Physiol,* 163, 1293-304.

BOUYER, D., GEIER, F., KRAGLER, F., SCHNITTGER, A., PESCH, M., WESTER, K., BALKUNDE, R., TIMMER, J., FLECK, C. & HULSKAMP, M. 2008. Two-dimensional patterning by a trapping/depletion mechanism: the role of TTG1 and GL3 in Arabidopsis trichome formation. *PLoS Biol,* 6, e141.

BRACHA-DRORI, K., SHICHRUR, K., KATZ, A., OLIVA, M., ANGELOVICI, R., YALOVSKY, S. & OHAD, N. 2004. Detection of protein-protein interactions in plants using bimolecular fluorescence complementation. *Plant J,* 40, 419-27.

BROSA, C. 1999. Biological effects of brassinosteroids. *Crit Rev Biochem Mol Biol,* 34, 339-58.

BROUN, P. 2005. Transcriptional control of flavonoid biosynthesis: a complex network of conserved regulators involved in multiple aspects of differentiation in Arabidopsis. *Curr Opin Plant Biol,* 8, 272-9.

CAMACHO, Y., MARTINEZ-CASTILLA, L., FRAGOSO, S., VAZQUEZ, S., MARTINEZ-BARAJAS, E. & COELLO, P. 2008. Characterization of a type A response regulator in the common bean (Phaseolus vulgaris) in response to phosphate starvation. *Physiol Plant,* 132, 272-82.

CANO-DELGADO, A., YIN, Y., YU, C., VAFEADOS, D., MORA-GARCIA, S., CHENG, J. C.,

NAM, K. H., LI, J. & CHORY, J. 2004. BRL1 and BRL3 are novel brassinosteroid receptors that function in vascular differentiation in Arabidopsis. *Development,* 131, 5341-51.

CEREZO, M., TILLARD, P., FILLEUR, S., MUNOS, S., DANIEL-VEDELE, F. & GOJON, A. 2001. Major alterations of the regulation of root NO(3)(-) uptake are associated with the mutation of Nrt2.1 and Nrt2.2 genes in Arabidopsis. *Plant Physiol,* 127, 262-71.

CHEN, C., ZOU, J., ZHANG, S., ZAITLIN, D. & ZHU, L. 2009. Strigolactones are a new-defined class of plant hormones which inhibit shoot branching and mediate the interaction of plant-AM fungi and plant-parasitic weeds. *Sci China C Life Sci,* 52, 693-700.

CHOE, S., DILKES, B. P., GREGORY, B. D., ROSS, A. S., YUAN, H., NOGUCHI, T., FUJIOKA, S., TAKATSUTO, S., TANAKA, A., YOSHIDA, S., TAX, F. E. & FELDMANN, K. A. 1999. The Arabidopsis dwarf1 mutant is defective in the conversion of 24-methylenecholesterol to campesterol in brassinosteroid biosynthesis. *Plant Physiol,* 119, 897-907.

CHOUNG, M. G., CHOI, B. R., AN, Y. N., CHU, Y. H. & CHO, Y. S. 2003. Anthocyanin profile of Korean cultivated kidney bean (Phaseolus vulgaris L.). *J Agric Food Chem,* 51, 7040-3.

CHUNG, Y., MAHARJAN, P. M., LEE, O., FUJIOKA, S., JANG, S., KIM, B., TAKATSUTO, S., TSUJIMOTO, M., KIM, H., CHO, S., PARK, T., CHO, H., HWANG, I. & CHOE, S. 2011. Auxin stimulates DWARF4 expression and brassinosteroid biosynthesis in Arabidopsis. *Plant J,* 66, 564-78.

CLOUSE, S. 2001. Brassinosteroids. *Curr Biol,* 11, R904.

CLOUSE, S. D. 1996. Plant hormones: brassinosteroids in the spotlight. *Curr Biol,* 6, 658-61.

CLOUSE, S. D., LANGFORD, M. & MCMORRIS, T. C. 1996. A brassinosteroid-insensitive mutant in Arabidopsis thaliana exhibits multiple defects in growth and development. *Plant Physiol,* 111, 671-8.

CLOUSE, S. D. & SASSE, J. M. 1998. BRASSINOSTEROIDS: Essential Regulators of Plant Growth and Development. *Annu Rev Plant Physiol Plant Mol Biol,* 49, 427-451.

CRIADO, M. V., CAPUTO, C., ROBERTS, I. N., CASTRO, M. A. & BARNEIX, A. J. 2009. Cytokinin-induced changes of nitrogen remobilization and chloroplast ultrastructure in wheat (Triticum aestivum). *J Plant Physiol,* 166, 1775-85.

DARE, A. P., SCHAFFER, R. J., LIN-WANG, K., ALLAN, A. C. & HELLENS, R. P. 2008. Identification of a cis-regulatory element by transient analysis of co-ordinately regulated genes. *Plant Methods,* 4, 17.

DE GRAUWE, L., VANDENBUSSCHE, F., TIETZ, O., PALME, K. & VAN DER STRAETEN, D. 2005. Auxin, ethylene and brassinosteroids: tripartite control of growth in the Arabidopsis hypocotyl. *Plant Cell Physiol,* 46, 827-36.

DE SAINT GERMAIN, A., BRAUN, N. & RAMEAU, C. 2010. [Strigolactones, a novel class of plant hormones controlling branching]. *Biol Aujourdhui,* 204, 43-9.

DECHORGNAT, J., NGUYEN, C. T., ARMENGAUD, P., JOSSIER, M., DIATLOFF, E., FILLEUR, S. & DANIEL-VEDELE, F. 2011. From the soil to the seeds: the long journey of nitrate in plants. *J Exp Bot,* 62, 1349-59.

DHAUBHADEL, S., BROWNING, K. S., GALLIE, D. R. & KRISHNA, P. 2002. Brassinosteroid functions to protect the translational machinery and heat-shock protein synthesis following thermal stress. *Plant J,* 29, 681-91.

DHAUBHADEL, S., CHAUDHARY, S., DOBINSON, K. F. & KRISHNA, P. 1999. Treatment with 24-epibrassinolide, a brassinosteroid, increases the basic thermotolerance of Brassica napus and tomato seedlings. *Plant Mol Biol,* 40, 333-42.

DIAZ, C., SALIBA-COLOMBANI, V., LOUDET, O., BELLUOMO, P., MOREAU, L., DANIEL-VEDELE, F., MOROT-GAUDRY, J. F. & MASCLAUX-DAUBRESSE, C. 2006. Leaf yellowing and anthocyanin accumulation are two genetically independent strategies in response to nitrogen limitation in Arabidopsis thaliana. *Plant Cell Physiol,* 47, 74-83.

DIVI, U. K., RAHMAN, T. & KRISHNA, P. 2010. Brassinosteroid-mediated stress tolerance in Arabidopsis shows interactions with abscisic acid, ethylene and salicylic acid pathways. *BMC Plant Biol,* 10, 151.

DUBROVSKY, J. G., SAUER, M., NAPSUCIALY-MENDIVIL, S., IVANCHENKO, M. G., FRIML, J., SHISHKOVA, S., CELENZA, J. & BENKOVA, E. 2008. Auxin acts as a local morphogenetic trigger to specify lateral root founder cells. *Proc Natl Acad Sci U S A,* 105, 8790-4.

EVANS, M. L. & ISHIKAWA, H. 1997. Cellular specificity of the gravitropic motor response in roots. *Planta,* 203, S115-22.

FEYISSA, D. N., LOVDAL, T., OLSEN, K. M., SLIMESTAD, R. & LILLO, C. 2009. The endogenous GL3, but not EGL3, gene is necessary for anthocyanin accumulation as induced by nitrogen depletion in Arabidopsis rosette stage leaves. *Planta,* 230, 747-54.

FONSECA, S., CHICO, J. M. & SOLANO, R. 2009. The jasmonate pathway: the ligand, the receptor and the core signalling module. *Curr Opin Plant Biol,* 12, 539-47.

FORDE, B. G. 2002a. Local and long-range signaling pathways regulating plant responses to nitrate. *Annu Rev Plant Biol,* 53, 203-24.

FORDE, B. G. 2002b. The role of long-distance signalling in plant responses to nitrate and other nutrients. *J Exp Bot,* 53, 39-43.

FRANCO-ZORRILLA, J. M., MARTIN, A. C., LEYVA, A. & PAZ-ARES, J. 2005. Interaction between phosphate-starvation, sugar, and cytokinin signaling in Arabidopsis and the roles of cytokinin receptors CRE1/AHK4 and AHK3. *Plant Physiol,* 138, 847-57.

FRANCO-ZORRILLA, J. M., MARTIN, A. C., SOLANO, R., RUBIO, V., LEYVA, A. &

PAZ-ARES, J. 2002. Mutations at CRE1 impair cytokinin-induced repression of phosphate starvation responses in Arabidopsis. *Plant J,* 32, 353-60.

FUJIOKA, S., LI, J., CHOI, Y. H., SETO, H., TAKATSUTO, S., NOGUCHI, T., WATANABE, T., KURIYAMA, H., YOKOTA, T., CHORY, J. & SAKURAI, A. 1997. The Arabidopsis deetiolated2 mutant is blocked early in brassinosteroid biosynthesis. *Plant Cell,* 9, 1951-62.

GANSEL, X., MUNOS, S., TILLARD, P. & GOJON, A. 2001. Differential regulation of the NO3- and NH4+ transporter genes AtNrt2.1 and AtAmt1.1 in Arabidopsis: relation with long-distance and local controls by N status of the plant. *Plant J,* 26, 143-55.

GAZZARRINI, S., LEJAY, L., GOJON, A., NINNEMANN, O., FROMMER, W. B. & VON WIREN, N. 1999. Three functional transporters for constitutive, diurnally regulated, and starvation-induced uptake of ammonium into Arabidopsis roots. *Plant Cell,* 11, 937-48.

GAZZARRINI, S. & MCCOURT, P. 2003. Cross-talk in plant hormone signalling: what Arabidopsis mutants are telling us. *Ann Bot,* 91, 605-12.

GINJOM, I. R., D'ARCY, B. R., CAFFIN, N. A. & GIDLEY, M. J. 2010. Phenolic contents and antioxidant activities of major Australian red wines throughout the winemaking process. *J Agric Food Chem,* 58, 10133-42.

GOJON, A., KROUK, G., PERRINE-WALKER, F. & LAUGIER, E. 2011. Nitrate transceptor(s) in plants. *J Exp Bot,* 62, 2299-308.

GOJON, A., NACRY, P. & DAVIDIAN, J. C. 2009. Root uptake regulation: a central process for NPS homeostasis in plants. *Curr Opin Plant Biol,* 12, 328-38.

GONZALEZ-GARCIA, M. P., VILARRASA-BLASI, J., ZHIPONOVA, M., DIVOL, F., MORA-GARCIA, S., RUSSINOVA, E. & CANO-DELGADO, A. I. 2011. Brassinosteroids control meristem size by promoting cell cycle progression in Arabidopsis roots. *Development,* 138, 849-59.

GONZALEZ, A., MENDENHALL, J., HUO, Y. & LLOYD, A. 2009. TTG1 complex MYBs, MYB5 and TT2, control outer seed coat differentiation. *Dev Biol,* 325, 412-21.

GONZALEZ, A., ZHAO, M., LEAVITT, J. M. & LLOYD, A. M. 2008. Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings. *Plant J,* 53, 814-27.

GRAY, W. M. 2004. Hormonal regulation of plant growth and development. *PLoS Biol,* 2, E311.

GROVE, M.D., SPENCER, G.F., & ROHWEDDER, W.K. 1979. Brassinolide, a plant growth-promoting steroid isolated from Brassica napus pollen. *Nature* 281, 216–217.

GUO, F. Q., WANG, R. & CRAWFORD, N. M. 2002. The Arabidopsis dual-affinity nitrate transporter gene AtNRT1.1 (CHL1) is regulated by auxin in both shoots and roots. *J Exp Bot,* 53, 835-44.

HAN, Y., VIMOLMANGKANG, S., SORIA-GUERRA, R. E., ROSALES-MENDOZA, S.,

ZHENG, D., LYGIN, A. V. & KORBAN, S. S. 2010. Ectopic expression of apple F3'H genes contributes to anthocyanin accumulation in the Arabidopsis tt7 mutant grown under nitrogen stress. *Plant Physiol,* 153, 806-20.

HE, J. X., GENDRON, J. M., SUN, Y., GAMPALA, S. S., GENDRON, N., SUN, C. Q. & WANG, Z. Y. 2005. BZR1 is a transcriptional repressor with dual roles in brassinosteroid homeostasis and growth responses. *Science,* 307, 1634-8.

HE, J. X., GENDRON, J. M., YANG, Y., LI, J. & WANG, Z. Y. 2002. The GSK3-like kinase BIN2 phosphorylates and destabilizes BZR1, a positive regulator of the brassinosteroid signaling pathway in Arabidopsis. *Proc Natl Acad Sci U S A,* 99, 10185-90.

HE, Z., WANG, Z. Y., LI, J., ZHU, Q., LAMB, C., RONALD, P. & CHORY, J. 2000. Perception of brassinosteroids by the extracellular domain of the receptor kinase BRI1. *Science,* 288, 2360-3.

HICHRI, I., BARRIEU, F., BOGS, J., KAPPEL, C., DELROT, S. & LAUVERGEAT, V. 2011. Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. *J Exp Bot,* 62, 2465-83.

HIROSE, N., TAKEI, K., KUROHA, T., KAMADA-NOBUSADA, T., HAYASHI, H. & SAKAKIBARA, H. 2008. Regulation of cytokinin biosynthesis, compartmentalization and translocation. *J Exp Bot,* 59, 75-83.

HOCH, W. A., SINGSAAS, E. L. & MCCOWN, B. H. 2003. Resorption protection. Anthocyanins facilitate nutrient recovery in autumn by shielding leaves from potentially damaging light levels. *Plant Physiol,* 133, 1296-305.

HOCH, W. A., ZELDIN, E. L. & MCCOWN, B. H. 2001. Physiological significance of anthocyanins during autumnal leaf senescence. *Tree Physiol,* 21, 1-8.

HU, Y., BAO, F. & LI, J. 2000. Promotive effect of brassinosteroids on cell division involves a distinct CycD3-induction pathway in Arabidopsis. *Plant J,* 24, 693-701.

HUNGRIA, M., JOSEPH, C. M. & PHILLIPS, D. A. 1991. Anthocyanidins and Flavonols, Major nod Gene Inducers from Seeds of a Black-Seeded Common Bean (Phaseolus vulgaris L.). *Plant Physiol,* 97, 751-8.

HUO, C. F., WANG, Z. Q., SUN, H. L., FAN, Z. Q. & ZHAO, X. M. 2008. [Interactive effects of light intensity and nitrogen supply on fraxinus mandshurica seedlings growth, biomass, and nitrogen allocation]. *Ying Yong Sheng Tai Xue Bao,* 19, 1658-64.

JIANG, C., GAO, X., LIAO, L., HARBERD, N. P. & FU, X. 2007. Phosphate starvation root architecture and anthocyanin accumulation responses are modulated by the gibberellin-DELLA signaling pathway in Arabidopsis. *Plant Physiol,* 145, 1460-70.

JING, P., BOMSER, J. A., SCHWARTZ, S. J., HE, J., MAGNUSON, B. A. & GIUSTI, M. M. 2008. Structure-function relationships of anthocyanins from various anthocyanin-rich extracts on the inhibition of colon cancer cell growth. *J Agric Food Chem,* 56, 9391-8.

KAGALE, S., DIVI, U. K., KROCHKO, J. E., KELLER, W. A. & KRISHNA, P. 2007.

Brassinosteroid confers tolerance in Arabidopsis thaliana and Brassica napus to a range of abiotic stresses. *Planta,* 225, 353-64.

KIBA, T., KUDO, T., KOJIMA, M. & SAKAKIBARA, H. 2011. Hormonal control of nitrogen acquisition: roles of auxin, abscisic acid, and cytokinin. *J Exp Bot,* 62, 1399-409.

KIEBER, J. J. & ECKER, J. R. 1993. Ethylene gas: it's not just for ripening any more! *Trends Genet,* 9, 356-62.

KIM, J., YI, H., CHOI, G., SHIN, B. & SONG, P. S. 2003. Functional characterization of phytochrome interacting factor 3 in phytochrome-mediated light signal transduction. *Plant Cell,* 15, 2399-407.

KIM, S. K., ABE, H., LITTLE, C. H. & PHARIS, R. P. 1990. Identification of Two Brassinosteroids from the Cambial Region of Scots Pine (Pinus silverstris) by Gas Chromatography-Mass Spectrometry, after Detection Using a Dwarf Rice Lamina Inclination Bioassay. *Plant Physiol,* 94, 1709-13.

KIM, S. Y., KIM, B. H., LIM, C. J., LIM, C. O. & NAM, K. H. 2010. Constitutive activation of stress-inducible genes in a brassinosteroid-insensitive 1 (bri1) mutant results in higher tolerance to cold. *Physiol Plant,* 138, 191-204.

KIM, T. W., GUAN, S., SUN, Y., DENG, Z., TANG, W., SHANG, J. X., BURLINGAME, A. L. & WANG, Z. Y. 2009. Brassinosteroid signal transduction from cell-surface receptor kinases to nuclear transcription factors. *Nat Cell Biol,* 11, 1254-60.

KRISHNA, P. 2003. Brassinosteroid-Mediated Stress Responses. *J Plant Growth Regul,* 22, 289-297.

KROUK, G., TILLARD, P. & GOJON, A. 2006. Regulation of the high-affinity NO3- uptake system by NRT1.1-mediated NO3- demand signaling in Arabidopsis. *Plant Physiol,* 142, 1075-86.

KUSHWAH, S., JONES, A. M. & LAXMI, A. 2011. Cytokinin interplay with ethylene, auxin and glucose signaling controls Arabidopsis seedling root directional growth. *Plant Physiol.*

LEA, U. S., SLIMESTAD, R., SMEDVIG, P. & LILLO, C. 2007. Nitrogen deficiency enhances expression of specific MYB and bHLH transcription factors and accumulation of end products in the flavonoid pathway. *Planta,* 225, 1245-53.

LEJAY, L., TILLARD, P., LEPETIT, M., OLIVE, F., FILLEUR, S., DANIEL-VEDELE, F. & GOJON, A. 1999. Molecular and functional regulation of two NO3- uptake systems by N- and C-status of Arabidopsis plants. *Plant J,* 18, 509-19.

LEWIS, D. R., RAMIREZ, M. V., MILLER, N. D., VALLABHANENI, P., RAY, W. K., HELM, R. F., WINKEL, B. S. & MUDAY, G. K. 2011. Auxin and ethylene induce flavonol accumulation through distinct transcriptional networks. *Plant Physiol,* 156, 144-64.

LEYSER, H. M. 1998. Plant hormones. *Curr Biol,* 8, R5-7.

LI, J. & CHORY, J. 1997. A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell,* 90, 929-38.

LI, J., NAGPAL, P., VITART, V., MCMORRIS, T. C. & CHORY, J. 1996. A role for brassinosteroids in light-dependent development of Arabidopsis. *Science,* 272, 398-401.

LI, J., NAM, K. H., VAFEADOS, D. & CHORY, J. 2001. BIN2, a new brassinosteroid-insensitive locus in Arabidopsis. *Plant Physiol,* 127, 14-22.

LI, L. & DENG, X. W. 2005. It runs in the family: regulation of brassinosteroid signaling by the BZR1-BES1 class of transcription factors. *Trends Plant Sci,* 10, 266-8.

LI, L., YE, H., GUO, H. & YIN, Y. 2010. Arabidopsis IWS1 interacts with transcription factor BES1 and is involved in plant steroid hormone brassinosteroid regulated gene expression. *Proc Natl Acad Sci U S A,* 107, 3918-23.

LI, Q. F., ZHANG, G. Y., DONG, Z. W., YU, H. X., GU, M. H., SUN, S. S. & LIU, Q. Q. 2009. Characterization of expression of the OsPUL gene encoding a pullulanase-type debranching enzyme during seed development and germination in rice. *Plant Physiol Biochem,* 47, 351-8.

LI, X., MO, X., SHOU, H. & WU, P. 2006. Cytokinin-mediated cell cycling arrest of pericycle founder cells in lateral root initiation of Arabidopsis. *Plant Cell Physiol,* 47, 1112-23.

LIANG, Y., MITCHELL, D. M. & HARRIS, J. M. 2007. Abscisic acid rescues the root meristem defects of the Medicago truncatula latd mutant. *Dev Biol,* 304, 297-307.

LILLO, C., LEA, U. S. & RUOFF, P. 2008. Nutrient depletion as a key factor for manipulating gene expression and product formation in different branches of the flavonoid pathway. *Plant Cell Environ,* 31, 587-601.

LIN, J. F. & WU, S. H. 2004. Molecular events in senescing Arabidopsis leaves. *Plant J,* 39, 612-28.

LOQUE, D., YUAN, L., KOJIMA, S., GOJON, A., WIRTH, J., GAZZARRINI, S., ISHIYAMA, K., TAKAHASHI, H. & VON WIREN, N. 2006. Additive contribution of AMT1;1 and AMT1;3 to high-affinity ammonium uptake across the plasma membrane of nitrogen-deficient Arabidopsis roots. *Plant J,* 48, 522-34.

LOVDAL, T., OLSEN, K. M., SLIMESTAD, R., VERHEUL, M. & LILLO, C. 2010. Synergetic effects of nitrogen depletion, temperature, and light on the content of phenolic compounds and gene expression in leaves of tomato. *Phytochemistry,* 71, 605-13.

MAES, L., INZE, D. & GOOSSENS, A. 2008. Functional specialization of the TRANSPARENT TESTA GLABRA1 network allows differential hormonal control of laminal and marginal trichome initiation in Arabidopsis rosette leaves. *Plant Physiol,* 148, 1453-64.

MANTELIN, S., DESBROSSES, G., LARCHER, M., TRANBARGER, T. J., CLEYET-MAREL, J. C. & TOURAINE, B. 2006. Nitrate-dependent control of root architecture and N nutrition are altered by a plant growth-promoting Phyllobacterium sp. *Planta,* 223, 591-603.

MASUCCI, J. D. & SCHIEFELBEIN, J. W. 1996. Hormones act downstream of TTG and GL2 to promote root hair outgrowth during epidermis development in the Arabidopsis root. *Plant Cell,* 8, 1505-17.

MATHUR, J., MOLNAR, G., FUJIOKA, S., TAKATSUTO, S., SAKURAI, A., YOKOTA, T., ADAM, G., VOIGT, B., NAGY, F., MAAS, C., SCHELL, J., KONCZ, C. & SZEKERES, M. 1998. Transcription of the Arabidopsis CPD gene, encoding a steroidogenic cytochrome P450, is negatively controlled by brassinosteroids. *Plant J,* 14, 593-602.

MAZZA, G. J. 2007. Anthocyanins and heart health. *Ann Ist Super Sanita,* 43, 369-74.

MI, G., CHEN, F. & ZHANG, F. 2008. Multiple signaling pathways control nitrogen-mediated root elongation in maize. *Plant Signal Behav,* 3, 1030-2.

MITCHELL, J. W. & GREGORY, L. E. 1972. Enhancement of overall plant growth, a new response to brassins. *Nat New Biol,* 239, 253-4.

MITCHELL, J. W., MANDAVA, N., WORLEY, J. F., PLIMMER, J. R. & SMITH, M. V. 1970. Brassins--a new family of plant hormones from rape pollen. *Nature,* 225, 1065-6.

MORA-GARCIA, S., VERT, G., YIN, Y., CANO-DELGADO, A., CHEONG, H. & CHORY, J. 2004. Nuclear protein phosphatases with Kelch-repeat domains modulate the response to brassinosteroids in Arabidopsis. *Genes Dev,* 18, 448-60.

MOUBAYIDIN, L., DI MAMBRO, R. & SABATINI, S. 2009. Cytokinin-auxin crosstalk. *Trends Plant Sci,* 14, 557-62.

MUSSIG, C. 2005. Brassinosteroid-promoted growth. *Plant Biol (Stuttg),* 7, 110-7.

NAHAR, K., KYNDT, T., DE VLEESSCHAUWER, D., HOFTE, M. M. & GHEYSEN, G. 2011. The jasmonate pathway is a key player in systemically induced defense against root knot nematodes in rice. *Plant Physiol.*

NAKAYA, M., TSUKAYA, H., MURAKAMI, N. & KATO, M. 2002. Brassinosteroids control the proliferation of leaf cells of Arabidopsis thaliana. *Plant Cell Physiol,* 43, 239-44.

NESI, N., JOND, C., DEBEAUJON, I., CABOCHE, M. & LEPINIEC, L. 2001. The Arabidopsis TT2 gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. *Plant Cell,* 13, 2099-114.

NETZEL, M., NETZEL, G., TIAN, Q., SCHWARTZ, S. & KONCZAK, I. 2006. Sources of antioxidant activity in Australian native fruits. Identification and quantification of anthocyanins. *J Agric Food Chem,* 54, 9820-6.

NOGUCHI, T., FUJIOKA, S., CHOE, S., TAKATSUTO, S., YOSHIDA, S., YUAN, H., FELDMANN, K. A. & TAX, F. E. 1999. Brassinosteroid-insensitive dwarf mutants of Arabidopsis accumulate brassinosteroids. *Plant Physiol,* 121, 743-52.

NOH, B. & SPALDING, E. P. 1998. Anion channels and the stimulation of anthocyanin accumulation by blue light in Arabidopsis seedlings. *Plant Physiol,* 116, 503-9.

OHKAMA, N., TAKEI, K., SAKAKIBARA, H., HAYASHI, H., YONEYAMA, T. &

FUJIWARA, T. 2002. Regulation of sulfur-responsive gene expression by exogenously applied cytokinins in Arabidopsis thaliana. *Plant Cell Physiol,* 43, 1493-501.

ONGARO, V. & LEYSER, O. 2008. Hormonal control of shoot branching. *J Exp Bot,* 59, 67-74.

PENG, M., HUDSON, D., SCHOFIELD, A., TSAO, R., YANG, R., GU, H., BI, Y. M. & ROTHSTEIN, S. J. 2008a. Adaptation of Arabidopsis to nitrogen limitation involves induction of anthocyanin synthesis which is controlled by the NLA gene. *J Exp Bot,* 59, 2933-44.

PENG, P., YAN, Z., ZHU, Y. & LI, J. 2008b. Regulation of the Arabidopsis GSK3-like kinase BRASSINOSTEROID-INSENSITIVE 2 through proteasome-mediated protein degradation. *Mol Plant,* 1, 338-46.

PLANCHET, E., RANNOU, O., RICOULT, C., BOUTET-MERCEY, S., MAIA-GRONDARD, A. & LIMAMI, A. M. 2011. Nitrogen metabolism responses to water deficit act through both abscisic acid (ABA)-dependent and independent pathways in Medicago truncatula during post-germination. *J Exp Bot,* 62, 605-15.

QI, T., SONG, S., REN, Q., WU, D., HUANG, H., CHEN, Y., FAN, M., PENG, W., REN, C. & XIE, D. 2011. The Jasmonate-ZIM-Domain Proteins Interact with the WD-Repeat/bHLH/MYB Complexes to Regulate Jasmonate-Mediated Anthocyanin Accumulation and Trichome Initiation in Arabidopsis thaliana. *Plant Cell.*

QUATTROCCHIO, F., WING, J. F., VAN DER WOUDE, K., MOL, J. N. & KOES, R. 1998. Analysis of bHLH and MYB domain proteins: species-specific regulatory differences are caused by divergent evolution of target anthocyanin genes. *Plant J,* 13, 475-88.

RAHMAN, A., HOSOKAWA, S., OONO, Y., AMAKAWA, T., GOTO, N. & TSURUMI, S. 2002. Auxin and ethylene response interactions during Arabidopsis root hair development dissected by auxin influx modulators. *Plant Physiol,* 130, 1908-17.

RAMANUJAM, P., FOGERTY, S., HEISER, W. & JOLLY, J. 1990. Fast gel electrophoresis to analyze DNA-protein interactions. *Biotechniques,* 8, 556-63.

REDIG, P., SHAUL, O., INZE, D., VAN MONTAGU, M. & VAN ONCKELEN, H. 1996. Levels of endogenous cytokinins, indole-3-acetic acid and abscisic acid during the cell cycle of synchronized tobacco BY-2 cells. *FEBS Lett,* 391, 175-80.

REINHARDT, D., MANDEL, T. & KUHLEMEIER, C. 2000. Auxin regulates the initiation and radial position of plant lateral organs. *Plant Cell,* 12, 507-18.

REVZIN, A. 1989. Gel electrophoresis assays for DNA-protein interactions. *Biotechniques,* 7, 346-55.

REYES, L. F. & CISNEROS-ZEVALLOS, L. 2003. Wounding stress increases the phenolic content and antioxidant capacity of purple-flesh potatoes (Solanum tuberosum L.). *J Agric Food Chem,* 51, 5296-300.

RICHARD-MOLARD, C., KRAPP, A., BRUN, F., NEY, B., DANIEL-VEDELE, F. & CHAILLOU, S. 2008. Plant response to nitrate starvation is determined by N storage

capacity matched by nitrate uptake capacity in two Arabidopsis genotypes. *J Exp Bot,* 59, 779-91.

RICHARD-MOLARD, C., WUILLEME, S., SCHEEL, C., GRESSHOFF, P. M., MOROT-GAUDRY, J. F. & LIMAMI, A. M. 1999. Nitrogen-induced changes in morphological development and bacterial susceptibility of belgian endive (Cichorium intybus L.) are genotype-dependent. *Planta,* 209, 389-98.

ROCK, C. D. & SUN, X. 2005. Crosstalk between ABA and auxin signaling pathways in roots of Arabidopsis thaliana (L.) Heynh. *Planta,* 222, 98-106.

ROMAN, G., LUBARSKY, B., KIEBER, J. J., ROTHENBERG, M. & ECKER, J. R. 1995. Genetic analysis of ethylene signal transduction in Arabidopsis thaliana: five novel mutant loci integrated into a stress response pathway. *Genetics,* 139, 1393-409.

ROWAN, D. D., CAO, M., LIN-WANG, K., COONEY, J. M., JENSEN, D. J., AUSTIN, P. T., HUNT, M. B., NORLING, C., HELLENS, R. P., SCHAFFER, R. J. & ALLAN, A. C. 2009. Environmental regulation of leaf colour in red 35S:PAP1 Arabidopsis thaliana. *New Phytol,* 182, 102-15.

RUBIO, V., BUSTOS, R., IRIGOYEN, M. L., CARDONA-LOPEZ, X., ROJAS-TRIANA, M. & PAZ-ARES, J. 2009. Plant hormones and nutrient signaling. *Plant Mol Biol,* 69, 361-73.

RYU, H., KIM, K., CHO, H. & HWANG, I. 2010. Predominant actions of cytosolic BSU1 and nuclear BIN2 regulate subcellular localization of BES1 in brassinosteroid signaling. *Mol Cells,* 29, 291-6.

SAKAKIBARA, H. 2003. Nitrate-specific and cytokinin-mediated nitrogen signaling pathways in plants. *J Plant Res,* 116, 253-7.

SAKAKIBARA, H., TAKEI, K. & HIROSE, N. 2006. Interactions between nitrogen and cytokinin in the regulation of metabolism and development. *Trends Plant Sci,* 11, 440-8.

SAKURAI, A. & FUJIOKA, S. 1997. Studies on biosynthesis of brassinosteroids. *Biosci Biotechnol Biochem,* 61, 757-62.

SALUK-JUSZCZAK, J. 2010. [Anthocyanins as components of functional food for cardiovascular risk prevention]. *Postepy Hig Med Dosw (Online),* 64, 451-8.

SCHMIDT, J., ALTMANN, T. & ADAM, G. 1997. Brassinosteroids from seeds of Arabidopsis thaliana. *Phytochemistry,* 45, 1325-7.

SCHMIDT, W., TITTEL, J. & SCHIKORA, A. 2000. Role of hormones in the induction of iron deficiency responses in Arabidopsis roots. *Plant Physiol,* 122, 1109-18.

SCHNITTGER, A., FOLKERS, U., SCHWAB, B., JURGENS, G. & HULSKAMP, M. 1999. Generation of a spacing pattern: the role of triptychon in trichome patterning in Arabidopsis. *Plant Cell,* 11, 1105-16.

SCHRAUT, D., HEILMEIER, H. & HARTUNG, W. 2005. Radial transport of water and abscisic acid (ABA) in roots of Zea mays under conditions of nutrient deficiency. *J Exp Bot,* 56,

879-86.

SCHUTZE, K., HARTER, K. & CHABAN, C. 2009. Bimolecular fluorescence complementation (BiFC) to study protein-protein interactions in living plant cells. *Methods Mol Biol,* 479, 189-202.

SCHWINN, K. & VENAIL, J. 2006. A Small Family of MYB-Regulatory Genes Controls Floral Pigmentation Intensity and Patterning in the Genus Antirrhinum. *The Plant Cell,* 18, 831–851.

SEGUELA, M., BRIAT, J. F., VERT, G. & CURIE, C. 2008. Cytokinins negatively regulate the root iron uptake machinery in Arabidopsis through a growth-dependent pathway. *Plant J,* 55, 289-300.

SHIMADA, Y., GODA, H., NAKAMURA, A., TAKATSUTO, S., FUJIOKA, S. & YOSHIDA, S. 2003. Organ-specific expression of brassinosteroid-biosynthetic genes and distribution of endogenous brassinosteroids in Arabidopsis. *Plant Physiol,* 131, 287-97.

SHIRLEY, B. W., KUBASEK, W. L., STORZ, G., BRUGGEMANN, E., KOORNNEEF, M., AUSUBEL, F. M. & GOODMAN, H. M. 1995. Analysis of Arabidopsis mutants deficient in flavonoid biosynthesis. *Plant J,* 8, 659-71.

SINGH, I. & SHONO, M. 2003. Effect of 24-epibrassinolide on pollen viability during heat-stress in tomato. *Indian J Exp Biol,* 41, 174-6.

SOLFANELLI, C., POGGI, A., LORETI, E., ALPI, A. & PERATA, P. 2006. Sucrose-specific induction of the anthocyanin biosynthetic pathway in Arabidopsis. *Plant Physiol,* 140, 637-46.

STADENBERG, I., MCDONALD, A. J. & PALMER, S. 1994. Diurnal variation in leaf extension of Salix viminalis at two nitrogen supply rates. *Tree Physiol,* 14, 1131-8.

SU, Y. H. & ZHANG, X. S. 2009. Auxin gradients trigger de novo formation of stem cells during somatic embryogenesis. *Plant Signal Behav,* 4, 574-6.

SUGIHARTO, B., BURNELL, J. N. & SUGIYAMA, T. 1992. Cytokinin Is Required to Induce the Nitrogen-Dependent Accumulation of mRNAs for Phosphoenolpyruvate Carboxylase and Carbonic Anhydrase in Detached Maize Leaves. *Plant Physiol,* 100, 153-6.

SUN, Y., FAN, X. Y., CAO, D. M., TANG, W., HE, K., ZHU, J. Y., HE, J. X., BAI, M. Y., ZHU, S., OH, E., PATIL, S., KIM, T. W., JI, H., WONG, W. H., RHEE, S. Y. & WANG, Z. Y. 2010. Integration of brassinosteroid signal transduction with the transcription network for plant growth regulation in Arabidopsis. *Dev Cell,* 19, 765-77.

SZEKERES, M., NEMETH, K., KONCZ-KALMAN, Z., MATHUR, J., KAUSCHMANN, A., ALTMANN, T., REDEI, G. P., NAGY, F., SCHELL, J. & KONCZ, C. 1996. Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in Arabidopsis. *Cell,* 85, 171-82.

TAKEI, K., TAKAHASHI, T., SUGIYAMA, T., YAMAYA, T. & SAKAKIBARA, H. 2002.

Multiple routes communicating nitrogen availability from roots to shoots: a signal transduction pathway mediated by cytokinin. *J Exp Bot,* 53, 971-7.

TAKEI, K., UEDA, N., AOKI, K., KUROMORI, T., HIRAYAMA, T., SHINOZAKI, K., YAMAYA, T. & SAKAKIBARA, H. 2004. AtIPT3 is a key determinant of nitrate-dependent cytokinin biosynthesis in Arabidopsis. *Plant Cell Physiol,* 45, 1053-62.

TANAKA, Y., SASAKI, N. & OHMIYA, A. 2008. Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *Plant J,* 54, 733-49.

TANG, W., KIM, T. W., OSES-PRIETO, J. A., SUN, Y., DENG, Z., ZHU, S., WANG, R., BURLINGAME, A. L. & WANG, Z. Y. 2008. BSKs mediate signal transduction from the receptor kinase BRI1 in Arabidopsis. *Science,* 321, 557-60.

TEALE, W. D., DITENGOU, F. A., DOVZHENKO, A. D., LI, X., MOLENDIJK, A. M., RUPERTI, B., PAPONOV, I. & PALME, K. 2008. Auxin as a model for the integration of hormonal signal processing and transduction. *Mol Plant,* 1, 229-37.

TEALE, W. D., PAPONOV, I. A. & PALME, K. 2006. Auxin in action: signalling, transport and the control of plant growth and development. *Nat Rev Mol Cell Biol,* 7, 847-59.

THOMMA, B. P., EGGERMONT, K., PENNINCKX, I. A., MAUCH-MANI, B., VOGELSANG, R., CAMMUE, B. P. & BROEKAERT, W. F. 1998. Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. *Proc Natl Acad Sci U S A,* 95, 15107-11.

THOMPSON, M. J., MANDAVA, N. B., MEUDT, W. J., LUSBY, W. R. & SPAULDING, D. W. 1981. Synthesis and biological activity of brassinolide and its 22 beta, 23 beta-isomer: novel plant growth-promoting steroids. *Steroids,* 38, 567-80.

TIAN, Q., CHEN, F., LIU, J., ZHANG, F. & MI, G. 2008. Inhibition of maize root growth by high nitrate supply is correlated with reduced IAA levels in roots. *J Plant Physiol,* 165, 942-51.

TITARENKO, E., ROJO, E., LEON, J. & SANCHEZ-SERRANO, J. J. 1997. Jasmonic acid-dependent and -independent signaling pathways control wound-induced gene activation in Arabidopsis thaliana. *Plant Physiol,* 115, 817-26.

TREHIN, C., PLANCHAIS, S., GLAB, N., PERENNES, C., TREGEAR, J. & BERGOUNIOUX, C. 1998. Cell cycle regulation by plant growth regulators: involvement of auxin and cytokinin in the re-entry of Petunia protoplasts into the cell cycle. *Planta,* 206, 215-24.

TSUCHIYA, Y. & MCCOURT, P. 2009. Strigolactones: a new hormone with a past. *Curr Opin Plant Biol,* 12, 556-61.

UESUSUKI, S., WATANABE, B., YAMAMOTO, S., OTSUKI, J., NAKAGAWA, Y. & MIYAGAWA, H. 2004. Synthesis of brassinosteroids of varying acyl side chains and evaluation of their brassinolide-like activity. *Biosci Biotechnol Biochem,* 68, 1097-105.

UMEHARA, M., HANADA, A., YOSHIDA, S., AKIYAMA, K., ARITE, T., TAKEDA-KAMIYA, N., MAGOME, H., KAMIYA, Y., SHIRASU, K., YONEYAMA,

K., KYOZUKA, J. & YAMAGUCHI, S. 2008. Inhibition of shoot branching by new terpenoid plant hormones. *Nature,* 455, 195-200.

VIDYA VARDHINI, B. & RAO, S. S. 2002. Acceleration of ripening of tomato pericarp discs by brassinosteroids. *Phytochemistry,* 61, 843-7.

VLOT, A. C., DEMPSEY, D. A. & KLESSIG, D. F. 2009. Salicylic Acid, a multifaceted hormone to combat disease. *Annu Rev Phytopathol,* 47, 177-206.

VON WIREN, N., GAZZARRINI, S., GOJON, A. & FROMMER, W. B. 2000. The molecular physiology of ammonium uptake and retrieval. *Curr Opin Plant Biol,* 3, 254-61.

WALCH-LIU, P., IVANOV, II, FILLEUR, S., GAN, Y., REMANS, T. & FORDE, B. G. 2006. Nitrogen regulation of root branching. *Ann Bot,* 97, 875-81.

WALKER, A. R., DAVISON, P. A., BOLOGNESI-WINFIELD, A. C., JAMES, C. M., SRINIVASAN, N., BLUNDELL, T. L., ESCH, J. J., MARKS, M. D. & GRAY, J. C. 1999. The TRANSPARENT TESTA GLABRA1 locus, which regulates trichome differentiation and anthocyanin biosynthesis in Arabidopsis, encodes a WD40 repeat protein. *Plant Cell,* 11, 1337-50.

WANG, T. W., COSGROVE, D. J. & ARTECA, R. N. 1993. Brassinosteroid Stimulation of Hypocotyl Elongation and Wall Relaxation in Pakchoi (Brassica chinensis cv Lei-Choi). *Plant Physiol,* 101, 965-968.

WANG, X., YI, K., TAO, Y., WANG, F., WU, Z., JIANG, D., CHEN, X., ZHU, L. & WU, P. 2006. Cytokinin represses phosphate-starvation response through increasing of intracellular phosphate level. *Plant Cell Environ,* 29, 1924-35.

WANG, Z. Y., NAKANO, T., GENDRON, J., HE, J., CHEN, M., VAFEADOS, D., YANG, Y., FUJIOKA, S., YOSHIDA, S., ASAMI, T. & CHORY, J. 2002. Nuclear-localized BZR1 mediates brassinosteroid-induced growth and feedback suppression of brassinosteroid biosynthesis. *Dev Cell,* 2, 505-13.

WANG, Z. Y., SETO, H., FUJIOKA, S., YOSHIDA, S. & CHORY, J. 2001. BRI1 is a critical component of a plasma-membrane receptor for plant steroids. *Nature,* 410, 380-3.

WINKEL-SHIRLEY, B. 2001. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiol,* 126, 485-93.

WIRTH, J., CHOPIN, F., SANTONI, V., VIENNOIS, G., TILLARD, P., KRAPP, A., LEJAY, L., DANIEL-VEDELE, F. & GOJON, A. 2007. Regulation of root nitrate uptake at the NRT2.1 protein level in Arabidopsis thaliana. *J Biol Chem,* 282, 23541-52.

YAN, Z., ZHAO, J., PENG, P., CHIHARA, R. K. & LI, J. 2009. BIN2 functions redundantly with other Arabidopsis GSK3-like kinases to regulate brassinosteroid signaling. *Plant Physiol,* 150, 710-21.

YE, Q., ZHU, W., LI, L., ZHANG, S., YIN, Y., MA, H. & WANG, X. 2010. Brassinosteroids control male fertility by regulating the expression of key genes involved in Arabidopsis anther and pollen development. *Proc Natl Acad Sci U S A,* 107, 6100-5.

YIN, Y., VAFEADOS, D., TAO, Y., YOSHIDA, S., ASAMI, T. & CHORY, J. 2005. A new class of transcription factors mediates brassinosteroid-regulated gene expression in Arabidopsis. *Cell,* 120, 249-59.

YIN, Y., WANG, Z. Y., MORA-GARCIA, S., LI, J., YOSHIDA, S., ASAMI, T. & CHORY, J. 2002. BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. *Cell,* 109, 181-91.

YU, X., LI, L., ZOLA, J., ALURU, M., YE, H., FOUDREE, A., GUO, H., ANDERSON, S., ALURU, S., LIU, P., RODERMEL, S. & YIN, Y. 2011. A brassinosteroid transcriptional network revealed by genome-wide identification of BESI target genes in Arabidopsis thaliana. *Plant J,* 65, 634-46.

YUAN, Y., CHIU, L. W. & LI, L. 2009. Transcriptional regulation of anthocyanin biosynthesis in red cabbage. *Planta,* 230, 1141-53.

ZHANG, H., RONG, H. & PILBEAM, D. 2007. Signalling mechanisms underlying the morphological responses of the root system to nitrogen in Arabidopsis thaliana. *J Exp Bot,* 58, 2329-38.

ZHAO, J., PENG, P., SCHMITZ, R. J., DECKER, A. D., TAX, F. E. & LI, J. 2002. Two putative BIN2 substrates are nuclear components of brassinosteroid signaling. *Plant Physiol,* 130, 1221-9.

ZHOU, A., WANG, H., WALKER, J. C. & LI, J. 2004. BRL1, a leucine-rich repeat receptor-like protein kinase, is functionally redundant with BRI1 in regulating Arabidopsis brassinosteroid signaling. *Plant J,* 40, 399-409.

ZHU, L. 1997. Yeast GAL4 two-hybrid system. A genetic system to identify proteins that interact with a target protein. *Methods Mol Biol,* 63, 173-96.

ZIMMERMANN, I. M., HEIM, M. A., WEISSHAAR, B. & UHRIG, J. F. 2004. Comprehensive identification of Arabidopsis thaliana MYB transcription factors interacting with R/B-like BHLH proteins. *Plant J,* 40, 22-34.