African Journal of Biotechnology Vol. 11(2), pp. 253-261, 5 January, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1825
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Probenazole treatment inhibits anthocyanins biosynthesis via salicylic acid and *AtNPR1* signal pathway in *Arabidopsis*

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Accepted 1 December, 2011

It has been found that anthocyanins were accumulated in *Arabidopsis* under drought or salt stress. In this study, such accumulation was found to be inhibited by external applied probenazole (3-allyloxy-1, 2-benzisothiazole-1,1-dioxide, PBZ), which is the active ingredient in oryzemate used for the protection of rice from *Magnaporthe grisea* (blast fungus). Microarray analysis indicated that expression of nine key genes in anthocyanins synthesis pathway was significantly repressed to about 4 folds lower by PBZ treatment. In transgenic *Arabidopsis* plants with a β-glucuronidase (GUS) reporter gene driven by a CaMV35S mini promoter combined with four salicylic acid-responsive elements, PBZ treatment resulted in increased expression of the GUS gene. When a salicylic acid (SA) synthesis mutant, *sid2-2*, and a SA signal transduction pathway mutant, *npr-1*, were treated with PBZ under the same drought condition, inhibition of anthocyanin accumulation was not observed. In addition, over-expression of NPR1 by CaMV35S promoter resulted in more inhibition on anthocyanins accumulation. Taken together, these results indicated that inhibition of anthocyanins accumulation by PBZ treatment is through SA and NPR1 signaling pathway in *Arabidopsis*.

Key words: Probenazole, anthocyanins biosynthesis, isochorismate synthase 1, nonexpressor of pathogenesis-related genes 1.

INTRODUCTION

Water stress can be induced in plant directly or indirectly by a number of environmental conditions. Plants exposed to drought, heat, cold, wind, flooding, or saline conditions often synthesize foliar anthocyanins in response (Ishikawa et al., 2005). Anthocyanins are water soluble pigments derived from flavonoids via the shikimic acid pathway. They are widely distributed in higher plant, and have been investigated extensively in the areas of chemistry, biochemistry and genetics (Hughes et al., 2010; Romero et al., 2008). There exist many correlative evidences for a relationship between anthocyanins and osmotic stress. Specifically, anthocyanins synthesis is known to be inducible under high salinity (Ichiyanagi et al., 2000; Rodrigues et al., 2006), drought (Castellarin et al., 2007; Hughes et al., 2010). The enzymes and genes involved in anthocyanins biosynthesis have been intensively investigated in model plant species (Tanaka et al., 2008). In *Arabidopsis*, the phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), dihydroflavonol reductase (DFR) and flavonoid 3-hydroxylase (F3H) genes have been found to be the essential components of the anthocyanins biosynthesis pathway (Boss et al., 1996; Romero et al., 2008; Xu and Li, 2006). Probenazole (3-allyloxy-1, 2-Benzisothiazole-1,1-dioxide, PBZ) is a highly effective chemical inducer of disease resistance (Sakamoto et al., 1999). It can prevent rice from the blast and pathogen infection in *Arabidopsis* and tobacco.

Extensive studies have demonstrated that PBZ and its active metabolite, 1,2-benzisothiazole-1, 1-dioxide (BIT),

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Abbreviations: PBZ, Probenazole; **SA,** salicylic acid; **GUS,** β-glucuronidase.

induces systemic acquired resistance (SAR) in *Arabidopsis* by stimulating the site upstream of salicylic acid (SA) accumulation during signal transduction (Iwai et al., 2007; Yoshioka et al., 2001). Many genes induced by PBZ have been identified recently (Zhao et al., 2007). NPR1 is a key regulator in the SAR signal transduction pathway. Upon SA treatment, NPR1 increases its transcription level, as well as translocate to the nucleus where it interacts with TGA factors, thereby involving in regulation of many defense-related genes expression. The promoters of plant genes are modular and contain a number of cis-acting elements, each of which may contribute to one or more aspects of a complex expression profile (Rushton et al., 2002). When taken out from their native promoter contexts, these cis-acting elements retain inducibility as components of other synthetic promoters in transgenic lines (Venter, 2007). One SA responsive element (SARE box) was isolated from upstream region of tobacco *PR2* gene. It is a cis-acting element, and it retained responses to SA treatment when combined with a 35S mini promoter (Shah and Klessig, 1996). Here a synthetic plant promoter containing defined SARE elements was used to identify the possible signaling transduction pathway between PBZ and anthocyanins accumulation.

In this study, we found that PBZ was able to inhibit anthocyanins accumulation in *Arabidopsis* under drought or salt stress. Microarray analysis, cis-elements transgenic assay and mutant analysis results indicated that SA signal transduction pathway may be involve in this inhibition. In addition, mutant analysis and over-expression of the NPR1 suggested that SA/NPR1 signal pathway plays a key role in anthocyanin biosynthesis.

MATERIALS AND METHODS

Seeds of *Arabidopsis thaliana* (L.) "Columbia" ("Col-0"wild type) and the mutant *sid2–2* were stratified at 4°C for 2 days. The germinates were grown in square pots (10-cm sides) containing a mixture of peat/vermiculite/perlite (3.0:9.0:0.5, v/v/v; Shanghai Institute of Landscape Science) that was pre-soaked with a MS (Murashige–Skoog) solution. All plants were placed in a growth room under an 8:16 h light/dark regimen (photosynthetic photon flux density of approximately 100 µmol $m^{-2}s^{-1}$), at 22 ± 2°C and 60% humidity. The 6-week old plants in same situation were used for the experiments.

Anthocyanin measurement

To estimate anthocyanin concentration, an entire rosette was ground in the square pots under a regular day/night cycle before the powder was mixed with 1 ml methanol-containing HCl and incubated for 24 h at 4°C in darkness. After centrifugation (13,000 × g, 10 min), the absorbance of the supernatant at 530 and 657 nm was measured. The content of anthocyanin was calculated as A_{530} - 0.25 A_{657}.

Drought stress, PBZ and SA treatment

To assess drought treatment, 6-week old soil-grown plants were irrigated with 0.5 mM PBZ or water. Then they were completely withheld from water for 16 days and re-watered at day 17 (Kang and Saltveit, 2002). For experimental variation, treatments and controls were grown in the same tray (15 plants per tray). Experiments were repeated at least three times. The seedlings were treated by drenching the roots and spraying the leaves with either plain water or the agricultural chemical oryzemate (Meijiseika), which contains 25% (wt/wt) probenazole. The efficient concentration used here was 0.5 mmol L^{-1}. After treatment, the leaves were harvested at indicated times. For SA treatment, 0.5 mmol L^{-1} was only used for spraying the leaves as reported (Loutfy et al., 2011).

Salt stress treatment

To examine salt resistance, 6-week old soil-grown plants were irrigated with 0.5 mM of PBZ or water. Then 7 days later, they were irrigated with 300 mM NaCl for 24 h. For experimental variation, treatments and controls were grown in the same tray (15 plants per tray). Experiments were repeated at least three times.

Affymetrix ATH1 GeneChip experiment

Wild-type plants were grown on soil for 4 weeks under an 8:16 h light/dark regimen at 22 ± 2°C with 60% humidity. Total RNA was prepared using TRIzol (Invitrogen, Carlsbad, CA, USA) with RNeasy mini kit (Qiagen) and multiple isopropanol precipitations. Affymetrix experiments were performed as described in the Affymetrix technical manual. Total RNA was used for cDNA and cRNA synthesis with the Affymetrix GeneChip One-Cycle Target Labeling Kit. Hybridizations, washing, staining and scanning were performed in Gene TechBio Technology Co., Ltd. Fold change for each gene was calculated by dividing the expression level of a PBZ treated 72 h sample by the expression level of a PBZ treated 0 h sample. A twofold difference in expression level between PBZ-treated 72 and 0 h samples were set as the threshold for considering a gene to be PBZ inducible. This experiment was repeated two times with two pairs of independent samples.

RNA analysis and real-time PCR

Total RNA was extracted from the leaves of water-treated or PBZ-treated WT and mutant *Arabidopsis*, using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). This was followed by chloroform-extraction and isopropanol precipitation. RNA samples were digested with RNAse-free DNase (Promega, Madison, WI, USA) and quantified by spectrophotometer. Afterward, 5 µg of total RNA was reverse-transcribed with SuperScript reverse transcriptase (Stratagene, La Jolla, CA, USA). The product was subsequently used as template. The extracted RNA was dissolved in 10 µL DEPC-treated water. First strand cDNA was synthesized using the SuperScript first-strand synthesis kit. After the cDNA synthesis reaction, RT-PCR was performed with 40 cycles of 95°C for 30 s, 60°C for 25 s, and 72°C for 20 s. Real-time polymerase chain reaction (PCR) was performed with SYBR Green I (TOYOBO Co., Osaka, Japan) on an iCycler (Bio-Rad, Hercules, CA, USA) according to the manufacturer's suggestions (Bio-Rad). The *Actin2 (ACT2)* gene served as a reference. Specific primers are listed in Table 1.

GUS assays

β-Glucuronidase (GUS) activity was determined using a fluorometric assay as described by Jefferson et al. (1987). The frozen leaves after different treatments were homogenized in 0.2 ml of chilled lysis buffer (0.1 M sodium phosphate pH 7.5, 10% glycerol

Table1. Oligonucleotide primers used in real-time reverse transcription (RT)-PCR.

Gene	Froward primer (5′–3′)	Reverse primer (5′–3′)
ACT2	GGCTCCTCTTAACCCAAAGGC	CACACCATCACCAGAATCCAG
PAL1	TCTCCTACATCGCCGGACTT	GACCTTCCTTAGGCTGGAGATCA
PAL2	GAACTGCGCCGATTCCTAAC	TCCTCTCCCGGAGACACAAC

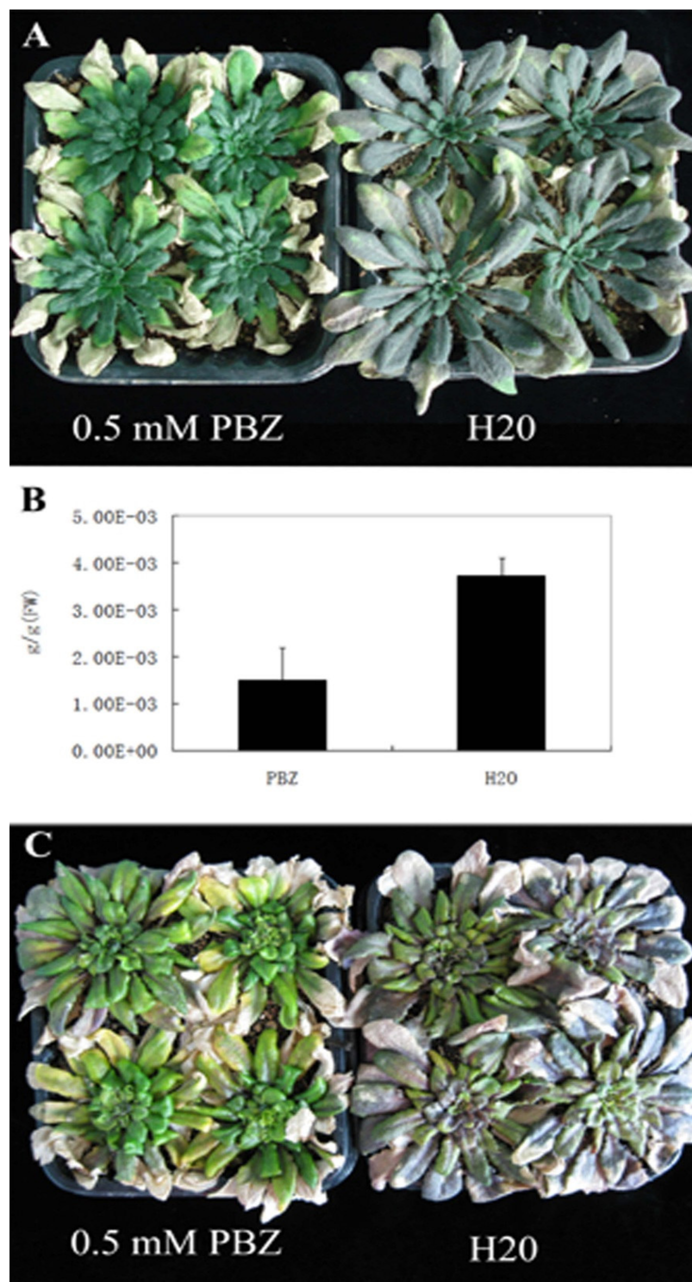

Figure 1. The basic experiment compared the growth phenotype of wild-type Columibia (Col)-0 plants upon anthocyanins biosynthesis with or without previous treatment with PBZ. (A) The growth phenotype of plants upon anthocyanins in drought stress. (B) The level of anthocyanin accumulation in un-treated and treated plants in drought stress. (C) The growth phenotype in 300 mM NaCl of wild-type Columibia (Col)-0 plants upon anthocyanins biosynthesis with or without previous treatment with PBZ.

and 1 mM EDTA) and 10 µL was used for measuring GUS activity. The reaction was terminated with sodium carbonate buffer and excited at 365 nm. The maximum emission was at 455 nm (Jefferson et al., 1987). GUS activity was normalized to protein concentration for each crude extract and calculated as pmol of 4-methlumbell-iferone produced per min per mg of soluble protein. Protein content was measured by the Bradford method using BSA as a standard.

RESULTS

Effect of PBZ treatment on anthocyanin accumulation under drought and salt conditions

To determine the effect of external PBZ application on anthocyanin accumulation under drought condition, wild-type (Col-0 ecotype) *Arabidopsis* plants were grown in plant growth soil in the presence of 0.5 mmol/L PBZ. As shown in Figure 1A, the un-treated plants clearly accumu-lated more brown pigments compared to PBZ treated plants. The level of anthocyanin accumulation in un-treated plants was 2.5-fold higher than those of treated plants (Figure 1B). Under salt stress with application of 300 mmol/L NaCl, similar result was obtained that un-treated plants showed more anthocyanin accumulation (Figure 1C). Thus, PBZ treatment was able to inhibit anthocyanin accumulation in *Arabidopsis* under drought and salt con-ditions.

Gene expression analysis

Microarray was used to identify genes which were involved in PBZ inhibited anthocyanin accumulation. Nine key genes including the PAL, CHS, CHI, DFR and F3H in anthocyanin synthesis pathway were significantly repres-sed to about 4 folds. Especially, the chalcone-flavanone isomerase gene was repressed to about 8 folds lower by PBZ treatment (Table 2). Q-RT-PCR was carried out for PAL1 and PAL2 genes, with ACT2 as reference. Expres-sion levels of PAL-1 and PAL-2 in plants treated for 0 day were normalized separately to “1”. The result obtained for quantitative real-time PCR was quite consistent with those data obtained via microarray data (Figure 2 and Table 2). Therefore, PBZ induced inhibition of anthocyanin accumulation via inhibiting the expression of a number of anthocyanin biosynthesis genes.

SARE cis-element transgenic plant analysis

Cis-element analysis was employed to test if SA pathway

Table 2. Microarray analysis of genes expression.

Gene ID	Annotation	Folder change (72 h vs 0 h)
AT5G13930	Chalcone synthase, CHS	-2.2
T3G55120	Chalcone isomerase, CHI	-1.9
AT5G05270	Chalcone flavanone isomerase	-2.7
AT5G08640	Flavonol synthase1, FLS1	-2.1
AT3G51240	Flavonol 3-hydroxylase, F3H	-2.2
AT1G65060	4-Coumaroy 1-CoA ligase 3, 4CL3	-2.3
AT3G19450	Cinnamyl-alcohol dehydrogenase, CAD	-1.2
AT2G37040	Phenylalanine ammonialyase1, PAL1	-1.8
AT3G53260	Phenylalanine ammonialyase2, PAL2	-1.6

Data from two independent replicates are presented. RNA samples were compared between Arabidopsis plants treated with PBZ for 72 h and 0 h. Note that fold-change values are log-base 2 transformed.

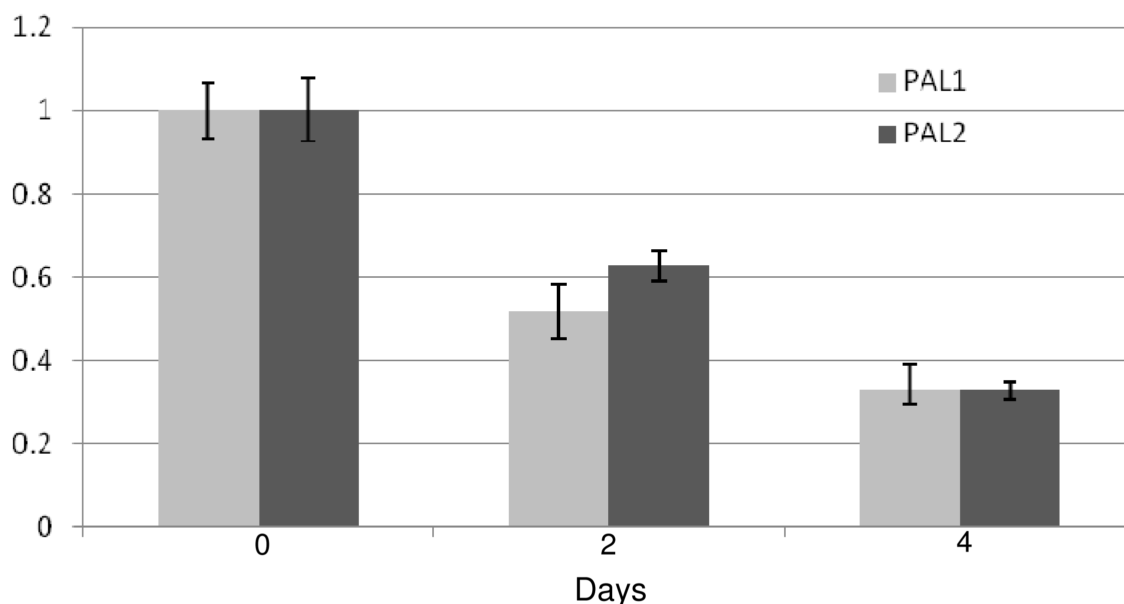

Figure 2. Real-time PCR analysis of expression for PAL1, PAL2. Total RNAs were obtained from leaves harvested at indicated days after treatment. Transcript levels were quantified by real-time RT-PCR, with ACT2 as reference. Expression level of each gene in plants treated for 0 day was normalized to “1”.

plays a role in PBZ-influence anthocyanins biosynthesis. Four SARE elements were inserted between the SpeI and XbaI sites in series in pCAMBIA1301 upstream of the -46 35S mini-promoter of Cauliflower mosaic virus (CaMV 35S). *Arabidopsis* plants were transformed with GUS driven by four salicylic acid-responsive elements and mini 46 bp 35SCaMV (*Cauliflower mosaic virus*) promoter (Figure 3A). To get homologous single copy transgenic plant, the GUS activity of the T3 transgenic lines was used for SA and PBZ treatment. The SARE transgenic plants were induced by SA as reported. When these lines were treated with 0.5 mM PBZ, the GUS activity was also up-regulated significantly (Figure 3B), while when treated by water, the GUS activity did not change. At the same time, the anthocyanin biosynthesis was inhibited as shown in Figure 1B. These results indicate that SA pathway may play a role in PBZ-inhibited anthocyanin biosynthesis process.

Anthocyanin accumulation assay on SA signal related mutants after PBZ treatment

PBZ can inhibit *Arabidopsis* anthocyanin biosynthesis, as well as up-regulated SA signal pathway. To investigate the relationship between SA and anthocyanin biosynthesis, we used the SA related mutants to learn their anthocyanin responses to drought stress. *sid2-2* is a salicylic acid (SA) synthesis mutant in which total SA accumulation is dramatically reduced. *npr1* is a SA signal transduction

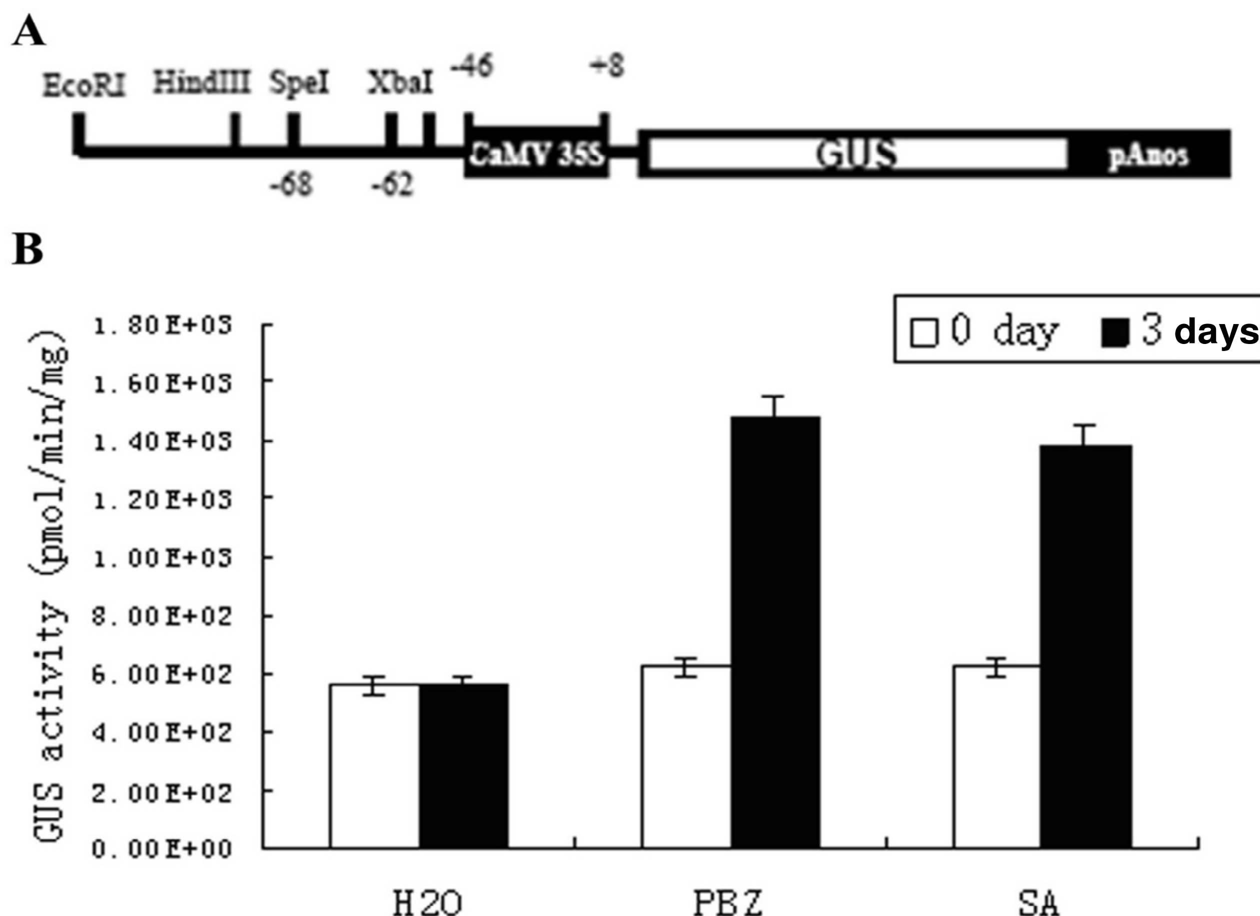

Figure 3. Inducible pattern of SARE cis-elements. (A) Scheme of the synthetic promoters. Elements were inserted between the SpeI and XbaI sites in pCAMBIA1301 upstream of the -46 35S mini-promoter of cauliflower mosaic virus (CaMV 35S). pAnos, nos terminator. (B) PBZ or salicylic acid inducibility of SARE transgenic T3 lines for 3 days.

pathway mutant, in which SA signal cannot be transduced to downstream genes. Compared to those of PBZ untreated controls, PBZ treated *sid2-2* exhibited even same phenotype on anthocyanin accumulation during drought stress (Figure 4A). Meanwhile, similar results were observed on mutant *npr1*. After treatment with 0.5 mmol/L PBZ, *npr1* exhibited little anthocyanin accumulation (Figure 4B and C). Similarly, *sid2-2* mutant also showed no obvious anthocyanin accumulation to PBZ treatment under salt stress condition (Figure 4D). Therefore, SA signals related mutant, *sid2-2* and *npr1*, showed natural anthocyanin biosynthesis after PBZ treatment during drought or salt stress. In other words, if no SA or its signal transduction, PBZ cannot inhibit anthocyanin biosynthesis.

Anthocyanin assay on *35S*::*NPR1*overexpression transgenic plant

To further confirm the relationship between anthocyanin biosynthesis and SA signal transduction pathway, we used a AtNPR1 over-expressed transgenic line, which contains the *AtNPR1* gene driven by cauliflower mosaic virus 35S promoter in Col-0 (*35S*::*NPR1*, given by Dr Dong X Nian and this line has a high expression of AtNPR1). Compared with the control, PBZ treated *35S*::*NPR1* showed more inhibition of anthocyanin biosynthesis after withholding irrigation for 16 days (Figure 5). The levels of anthocyanin accumulated in *35S*::*NPR1* treated with 0.5 mmol/L PBZ were 4.3-fold lower than untreated controls. Compared to wild type plant in anthocyanin accumulation, *35S*::*NPR1* transgenic plants exhibited 2.5-fold lower in untreated situation and slightly lower after PBZ treatment. It means that if the SA signal transduction pathway was constitutively activated, it could inhibit anthocyanin accumulation by itself, and would enhance anthocyanin biosynthesis inhibition by PBZ treatment.

DISCUSSION

Drought or salt treatments in this study are different types

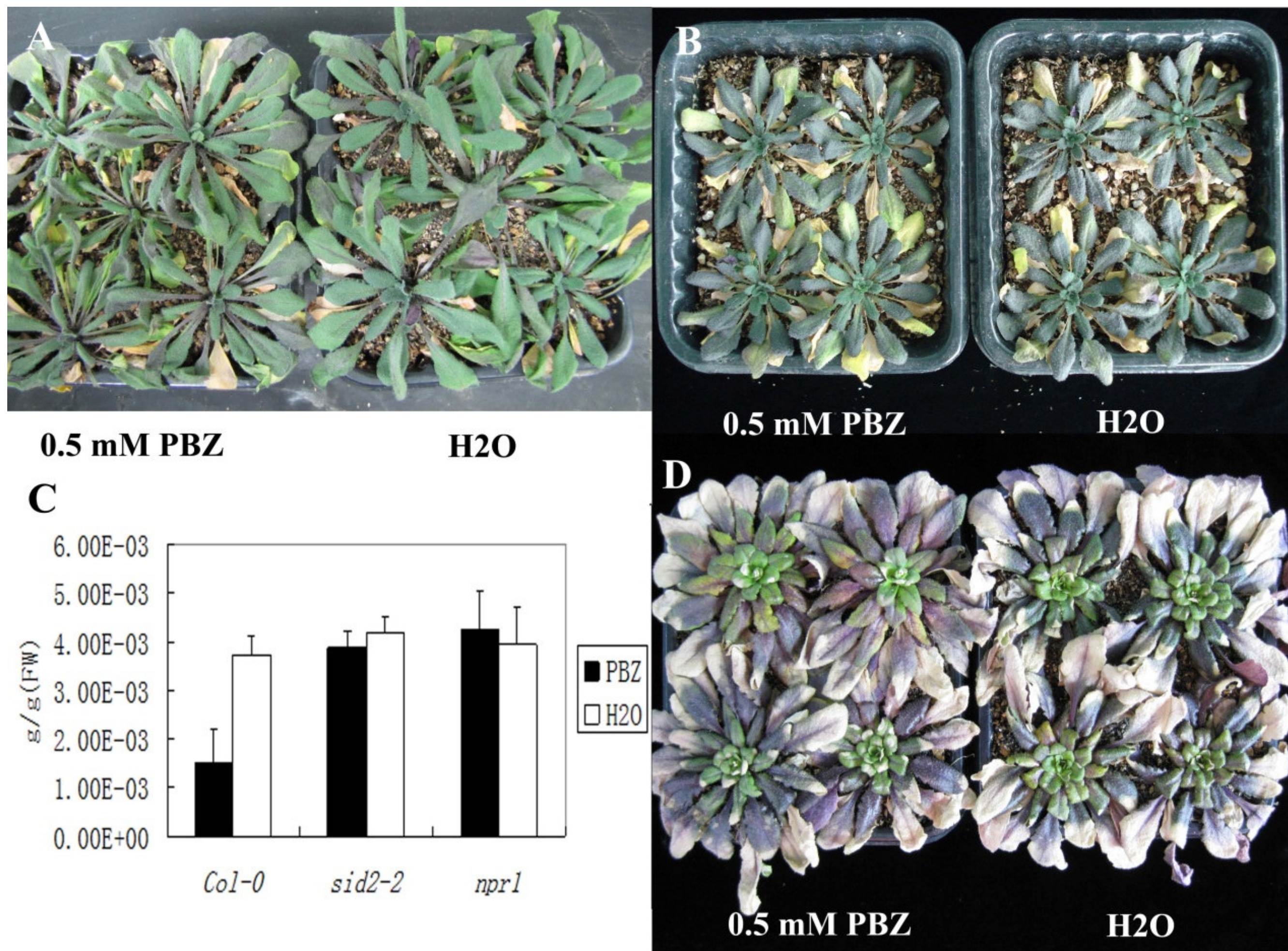

Figure 4. Phenotype of various SA signals relative mutants after treatment with or without PBZ in drought (by withholding irrigation for 16 days) and salt (300 mM NaCl) stress condition. (A) Phenotype of *sid2-2* after treatment with or without PBZ to drought stress (by withholding irrigation for 16 days). (B) Phenotype of *npr1* after treatment with or without PBZ to drought stress (by withholding irrigation for 16 days). (C) The level of anthocyanin accumulation in un-treated and treated plants, including *sid2-2* and *npr1* in drought stress condition. (D) Phenotype of *sid2-2* after treatment with or without PBZ to salt stress (300 mM NaCl).

of low osmotic stress which is generally indicative of an increased accumulation of solutes (Verslues et al., 2006). In addition to lower osmotic potential, red-leafed changes by anthocyanin accumulation also will make cell walls harder (lower elasticity) significantly than green leaves (Castellarin et al., 2007; Taulavuori et al., 2010). This phenomenon may allow anthocyanins-containing leaves to tolerate suboptimal water levels. The often transitory nature of foliar anthocyanins accumulation may allow plants to respond quickly and temporarily to environmental variability rather than through more permanent anatomical or morphological modifications (Hughes et al., 2010). Induction of plant disease resistance by PBZ has been well studied, but no data suggests it has relationship with anthocyanin accumulation (Iwai et al., 2007; Zhao et al., 2007). In this study, we observed PBZ treatment could inhibit anthocyanin accumulation during drought or salt stress. Through microarray analysis, many anthocyanin synthesis genes were identified to be inhibited by PBZ treatment. Therefore, the anthocyanin accumulation inhibition phenotype should come from inhibition of anthocyanin biosynthesis by PBZ.

SARE is a salicylic acid-responsive element in the promoter of the tobacco pathogenesis-related β-1,3-glucanase gene, *PR-2d,* and it retained responses to SA treatment when combined with a 35S mini promoter (Shah and Klessig, 1996). By SARE cis-element transgenic plant analysis, PBZ was found to inhibit *Arabidopsis* anthocyanin biosynthesis as well as up-regulate SA signal pathway at the same time. This data gives a clue to test the relationship between anthocyanin biosynthesis and SA signal transduction pathway. SA acts as a potential non-enzymatic antioxidant, as well as a plant growth regulator, which plays an important role in regulating a number of plant physiological processes including photosynthesis (Loutfy et al., 2011; Rivas-San Vicente and

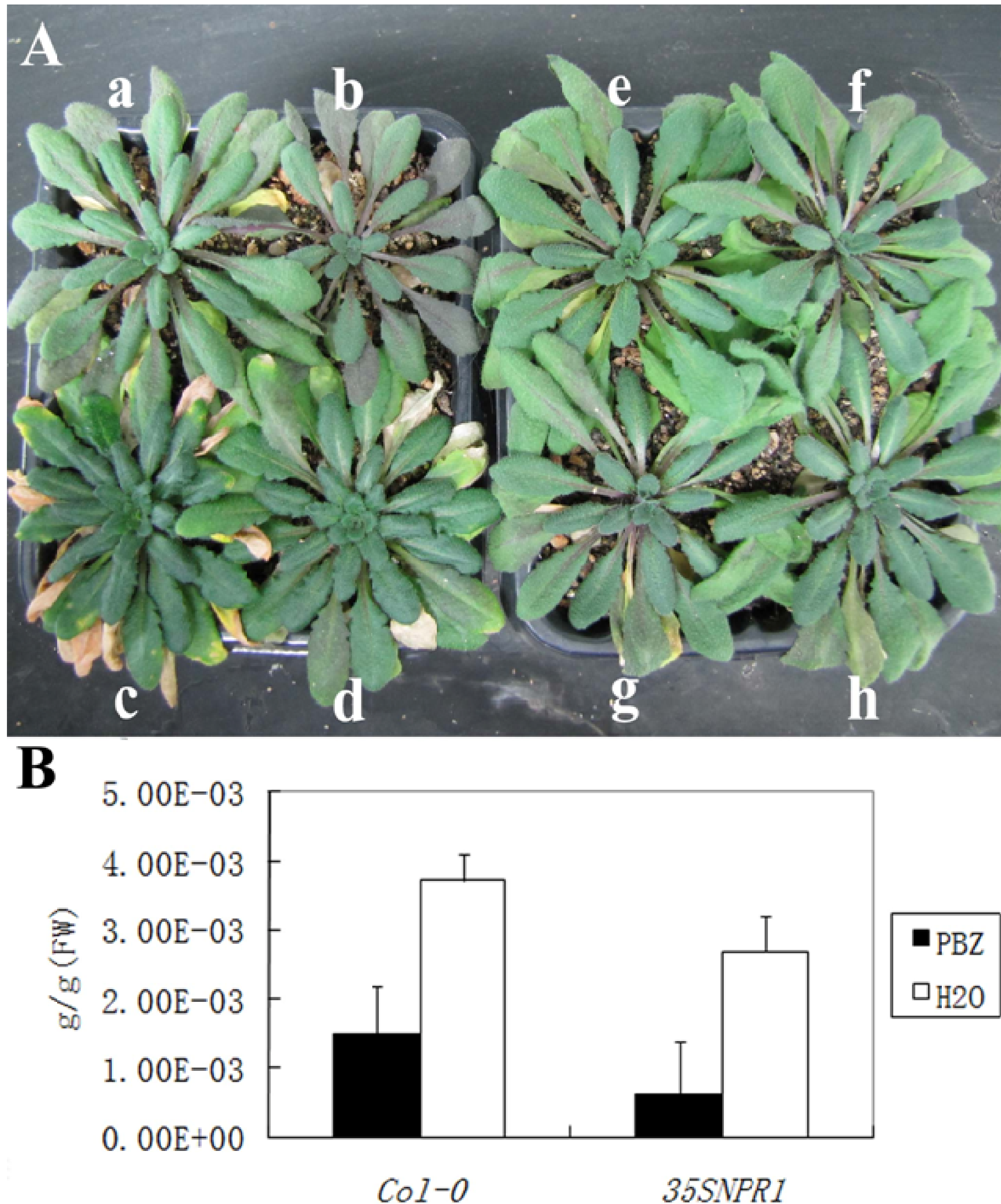

Figure 5. Phenotype of *Arabidopsis* plants to anthocyanins accumulation after PBZ or water treatment during drought stress. (A) The growth phenotype of plants upon anthocyanins in drought stress. (a) Response of PBZ treated plants (*sid2-2*) to drought stress (by withholding irrigation for 16 days). (b) Response of PBZ treated plants (*npr1*) to drought stress (by withholding irrigation for 16 days). (c) Response of PBZ treated plants (*35S*::*NPR1*) to drought stress (by withholding irrigation for 16 days). (d) Response of PBZ treated plants (Col-0) to drought stress (by withholding irrigation for 16 days). (e) Response of water treated plants (*sid2-2*) to drought stress (by withholding irrigation for 16 days). (f) Response of water treated plants (*npr1*) to drought stress (by withholding irrigation for 16 days). (g) Response of water treated plants (*35S*::*NPR1*) to drought stress (by withholding irrigation for 16 days). (h) Response of water treated plants (Col-0) to drought stress (by withholding irrigation for 16 days). (B) The level of anthocyanin accumulation in un-treated and treated plants in drought stress.

Plasencia, 2011; Vasiukova and Ozeretskovskaia, 2007). Some earlier reports showed that exogenous SA could alleviate the damaging effects of drought stress (Munne-Bosch and Penuelas, 2003; Taulavuori et al., 2010) and salt stress in wheat (Arfan et al., 2007), while no relationship was reported between SA and anthocyanin accumulation. To further learn such a relationship, SA related mutants, *sid2-2* and *npr1*, together with AtNPR1 over expression transgenic plants were also employed. For the SA related mutants with or without PBZ treatment, anthocyanin biosynthesis was not inhibited during drought or salt stress. Compared with SA related mutants and wild type plant, PBZ treated *35S*::*NPR1* showed more inhibition of anthocyanin biosynthesis after withholding irrigation. Therefore, these results showed that PBZ treatment could inhibit anthocyanin biosynthesis by SA synthesis and NPR1 signaling pathway in *Arabidopsis*.

Interestingly, although PBZ inhibited anthocyanin biosynthesis using SA signal transduction pathway, PBZ treated plant have more drought and salt stress tolerance and similar results was observed in our experiment (data not shown). This meant that anthocyanin accumulation is not always accompanied with drought and salt stress, and this loose link can be broken down by PBZ treatment. In nature, both red and green-leafed groups contain species exhibiting a broad range of drought tolerance (Hughes et al., 2010). And plants have many molecular control mechanisms for abiotic stress tolerance, which are based on the activation and regulation of specific stress related genes. These genes are involved in the whole process of stress responses, such as signaling, transcriptional control, protection of membranes and proteins, and free-radical and toxic-compound scavenging. Anthocyanin is only one of the strategies used by plant during drought or salt stress (Ashraf, 2010; Ramachandra Reddy et al., 2004). Regardless of the developmental or environmental signal responsible of anthocyanin accumulation, it also can represent a significant metabolic cost to the plant. Energy is required to modify flavononal precursors and form anthocyanin *de novo* (Ishikawa et al., 2005).

Furthermore, potential cost of anthocyanin accumulation is the resulting interference with the light reactions of photosynthesis because of their ability to absorb blue and reflect red wave lengths. Reductions in photosynthetic rates have been noted in red-leafed varieties of Coleus and *Capsicum annuum* (Romero et al., 2008). Therefore, there should be some precise mechanism to regulate anthocyanin accumulation so that an elaborate balance can be kept for plant to respond to environmental change properly. In this study, PBZ treatment inhibited anthocyanin accumulation by activating internal SA signal pathway, which is a precise regulation mechanism. Besides this, NPR1 overexpression lines displayed lower anthocyanin accumulation after PBZ treatment. This result shows that besides nuclear location, expression level can also influence NPR1 function. However, it remains unknown which detailed signaling components, in addition to NPR1, participate in regulation and how SA/NPR1 pathway influences anthocyanin biosynthesis. Additional studies will therefore focus on elucidation of the detailed components and the mechanism involved in PBZ treatment system. The application of PBZ for anthocyanin biosynthesis control in other crops can also be further researched.

In conclusion, this is the first report stating that anthocyanin accumulation can be inhibited by PBZ treatment. And we initially identified that SA signal transduction pathway plays a pivotal role during this process in *Arabidopsis*. This may provide a key to understanding the molecular basis for modulating anthocyanin accumulation during drought and salt stress and could help researchers draw a universal anthocyanin accumulation regulation map. Besides, it will also help researchers learn more about PBZ-plant interaction mechanism and find more application of PBZ on other crops.

ACKNOWLEDGEMENTS

We are grateful to Professor X. Dong for providing the *sid2-2* mutant and *35S*::*NPR1*. This work was supported by grants from the National Program of Transgenic Variety Development of China (2008ZX08009-004, 2009ZX08009-061B) and from the Science and Technology Committee of the Shanghai Municipal Government (Grant No. 08DZ2270800).

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