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Abscisic acid-induced hydrogen peroxide is required for anthocyanin accumulation in leaves of rice seedlings

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Summary

The role of hydrogen peroxide (H_2O_2) in abscisic acid (ABA)-induced anthocyanin accumulation in detached and intact leaves of rice seedlings was investigated. Treatment with ABA resulted in an accumulation of anthocyanins in detached rice leaves. Dimethylthiourea, a chemical trap for H_2O_2, was observed to be effective in inhibiting ABA-induced accumulation of anthocyanins. Inhibitors of NADPH oxidase (diphenyleneiodonium chloride and imidazole), phosphatidylinositol 3-kinase (wortmannin and LY 294002), and a donor of nitric oxide (*N-tert*-butyl-α-phenylnitrone), which have previously been shown to prevent ABA-induced H_2O_2 accumulation in detached rice leaves, inhibited ABA-induced anthocyanin increase. Exogenous application of H_2O_2, however, was found to increase the anthocyanin content of detached rice leaves. In terms of H_2O_2 accumulation, intact (attached) leaves of rice seedlings of cultivar Taichung Native 1 (TN1) are ABA sensitive and those of cultivar Tainung 67 (TNG67) are ABA insensitive. Upon treatment with ABA, H_2O_2 and anthocyanins accumulated in leaves of TN1 seedlings but not in leaves of TNG67. Our results, obtained from detached and intact leaves of rice seedlings, suggest that H_2O_2 is involved in ABA-induced anthocyanin accumulation in this species.

Introduction

Anthocyanins, such as delphinidin, cyanidin, and pelargonidin, are water-soluble pigments found in all plant tissues through the plant kingdom and synthesized through the phenylpropanoid and flavonoid pathways (Holton and Cornish, 1995).

Abbreviations: ABA, abscisic acid; c-PTIO, 2-(4-carboxy-2-phenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide; DMTU, dimethylthiourea; DPI, diphenyleneiodonium chloride; IMD, imidazole; LY, LY 294002; NO, nitric oxide; PAL, phenylalanine ammonia-lyase; PBN, *N-tert*-butyl-α-phenylnitrone; ROS, reactive oxygen species; TN1, Taichung Native 1; TNG67, Tainung 67; WM, wortmannin.

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Flavonoids are derived from the aromatic amino acid phenylalanine via cinnamic and coumaric acids. Phenylalanine ammonia-lyase (PAL, EC 4. 3. 1. 5), a key regulatory enzyme of anthocyanin biosynthesis from phenylalanine, is synthesized *de novo* in many plant tissues. It has been suggested that anthocyanins have antioxidant functions, posses antifugal/antibiotic capabilities, serve a photoprotective role, and allow the plants to develop resistance to environmental stresses, such as UVB radiation, drought, and cold (Chalker-Scott, 1999).

It has been shown that the production of anthocyanins in leaves of *Arabidopsis thaliana*, *Cornus stolonifera*, *Vigna unguiculata*, *and Zea mays*, and fruits of *Vitis vinifera* can be induced or up-regulated by low temperatures (Christie et al., 1994; Pietrini and Massacci, 1998; Kubo et al., 1999), drought (Balakumar et al., 1993; Castellarin et al., 2007), and senescence (Field et al., 2001; Hoch et al., 2001). These stresses are known to cause accumulation of abscisic acid (ABA) in plant tissues (Gepstein and Thimann, 1980; Zeevaart, 1980 Lee et al., 1993; Munns and Sharp, 1993; Montero et al., 1997; Yang et al., 2002; Aroca et al., 2003; Xiong and Zhu, 2003).

Fambrini et al. (1993) demonstrated that a sunflower mutant, deficient in carotenoid synthesis and ABA levels, prevents anthocyanin synthesis. Paek et al. (1997) reported that ABA-deficient *Viviparous-1* (*Vp1*) mutants of maize kernels still accumulate anthocyanins. Fluridone is known to inhibit phytoene desaturation and suppress endogenous ABA synthesis (Bartels and Watson, 1978). When developing *Vp1* kernels were cultured on fluridone to reduce endogenous ABA to near zero levels, anthocyanin synthesis was completely repressed (Paek et al., 1997). Addition of exogenous ABA to fluridone medium induced anthocyanin synthesis in *Vp1* kernels (Paek et al., 1997). Recent results also suggest that changes in the content of endogenous ABA may play an important role in the induction of anthocyanin synthesis in regenerated *Torenia fournieri* shoots (Nagira et al., 2006). Effects of exogenous ABA on anthocyanin accumulation have also been examined. Elevated anthocyanin level has been measured in plants treated with ABA (Pirie and Mullins, 1976; Jiang and Joyce, 2003; Jeong et al., 2004). However, there are reports indicating that anthocyanin level decreased in plants treated with ABA (Guruprasad and Laloraya, 1980; Ozeki and Komamine, 1986). Ithal and Reddy (2004) provided evidence to show that anthocyanin accumulation in rice leaves in response to 100 μmol L^{-1} ABA is genotype-dependent.

Recently, many investigators have focused on the functional aspects of H_2O_2. H_2O_2 is a major reactive oxygen species (ROS) generated in plants, which is scavenged by a network of low molecular weight antioxidants and antioxidant enzymes (Asada, 1999). Because H_2O_2 is relatively stable and diffusible through membrane, it is generally thought to serve as a signal molecule under various abiotic stresses (Neill et al., 2002), in acclimation to photo-oxidative stress (Karpinski et al., 1999), in plant–pathogen interactions (Levine et al., 1994), and in ABA-induced stomatal closure (Zhang et al., 2001).

We have demonstrated previously that ABA increases the activity of PAL, a key regulatory enzyme of anthocyanin biosynthesis, and promotes senescence in rice leaves (Hung and Kao, 2004, 2005a). Thus, anthocyanin content in rice leaves is expected to be increased by ABA. Our previous work also showed that H_2O_2 is involved in ABA-increased PAL activity and ABA-promoted senescence in rice leaves (Hung and Kao, 2004, 2005a). Here we have examined the possible involvement of H_2O_2 in ABA-induced anthocyanin accumulation in rice leaves.

Materials and methods

Plant materials

Rice (*Oryza sativa* L., cv. Taichung Native 1 (TN1) or Tainung 67 (TNG67)) seeds were sterilized with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water. These seeds were then germinated in Petri dishes with wetted filter paper at 37 °C under dark conditions. After 48 h of incubation, uniformly germinated seeds were selected and cultivated in a 500 mL beaker containing half-strength Kimura B solution as described previously (Hsu and Kao, 2005). The hydroponically cultivated seedlings were grown for 12 d in a Phytotron with natural sunlight at 30 °C day/25 °C night and 90% relative humidity. The apical 3 cm of the third leaf was used in all experiments. A group of 10 segments was floated in a Petri dish containing 10 mL of test solution. Incubation was carried out at 27 °C in the dark. In experiments with intact leaves of TN1 and TNG6 7 seedlings, ABA was added to half-strength Kimura B solution at the time when the third leaf was fully expanded.

Determination of anthocyanins and H_2O_2

Quantitative analysis of anthocycnins was performed spectrophotometrically (Kubo et al., 1999). Leaf samples were homogenized with potassium phosphate buffer (pH 7.8, 100 mmol L^{-1}). Our preliminary experiments demonstrated that the absorbance of crude rice leaf extract

peaks at 600 nm. Kubo et al. (1999) claimed that the increase in absorbance at 600 nm is thought to be due mainly to accumulation of anthocyanins. Thus, for anthocyanin determination, the absorbance of the leaf extract at 600 nm was measured with a UV-2800 spectrophotometer (Hitachi, Tokyo, Japan). One absorbance unit was defined as the amount of anthocyanins giving an absorbance of 0.1 at 600 nm. The H_2O_2 content was measured colorimetrically as described by Jana and Choudhuri (1982). H_2O_2 was extracted by homogenizing leaf tissue with phosphate buffer (50 mmol L^{-1}, pH 6.5) containing 1 mmol L^{-1} hydroxylamine. The homogenate was centrifuged at 6000 g_n for 25 min. To determine H_2O_2 content, the extracted solution was mixed with 0.1% titanium chloride in 20% (v/v) H_2SO_4. The mixture was then centrifuged at 6000g_n for 25 min. The absorbance was measured at 410 nm. Using this method, we determined that absorbance increased linearly with the amount of H_2O_2, and addition of H_2O_2 to extracts resulted in the predicted increase of absorbance, i.e. added H_2O_2 was fully recovered. The H_2O_2 content was calculated using an extinction coefficient 0.28 $\mu mol^{-1} cm^{-1}$. The contents of anthocyanins and H_2O_2 were expressed on the basis of initial fresh weight.

Statistical analysis

Statistical differences between measurements ($n = 4$) on different treatments or at different times were analyzed following Duncan's multiple range test or Student's *t*-test.

Results and discussion

ABA increases anthocyanin content

Anthocyanin content in the control leaves remained unchanged during the first 2 of incubation in the dark and increased subsequently (Figure 1). It is clear that ABA-treated rice leaves had higher anthocyanin contents than the control leaves at 2 and 3 d after treatment (Figure 1).

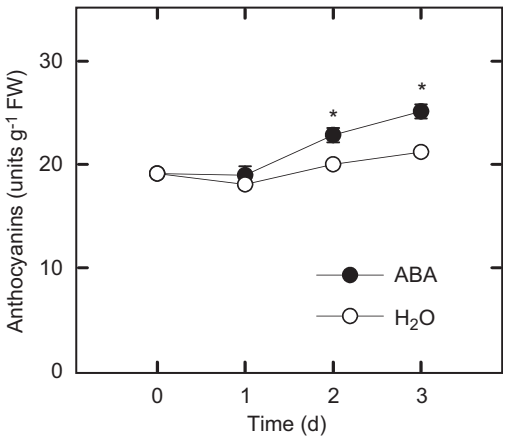

Figure 1. Changes in anthocyanin content in rice leaves treated with either water or 45 $\mu mol\ L^{-1}$ ABA in the dark. Vertical bars represent standard errors ($n = 4$). Asterisks represent values that are significant at $P<0.05$ by Student's *t*-test when compared with water control.

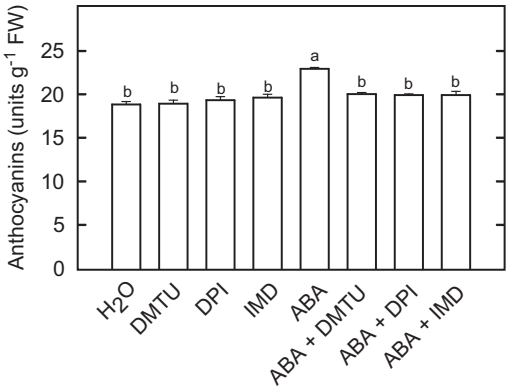

Figure 2. Effect of DMTU, DPI, and IMD on anthocyanin content in rice leaves treated with ABA. The concentrations of ABA, DMTU, DPI, and IMD were 45 $\mu mol\ L^{-1}$, 5 mmol L^{-1}, 25 $\mu mol\ L^{-1}$, and 100 $\mu mol\ L^{-1}$, respectively. All measurements were determined 2 d after treatment in the dark. Vertical bars represent standard errors ($n = 4$). Values with the same letter are not significantly different at $P<0.05$, according to Duncan's multiple range test.

Effect of dimethylthiourea (DMTU), a chemical trap for H_2O_2 and NADPH oxidase inhibitors

We have previously shown that ABA-induced H_2O_2 production in rice leaves is evident 1 d after treatment (Hung and Kao, 2004). In several plant systems, H_2O_2 has been shown to function as a signal molecule (Levine et al., 1994; Rao et al., 1997; Karpinski et al., 1999; Casano et al., 2001; Zhang et al., 2001; Neill et al., 2002). It seems that the accumulation of H_2O_2 in rice leaves induced by ABA may play an important role in regulating the increase in anthocyanin content in rice leaves. To test this hypothesis, DMTU, a chemical trap for H_2O_2 (Levine et al., 1994; Rao et al., 1997; Casano et al., 2001), was used. As indicated in Figure 2, ABA-induced anthocyanin accumulation was significantly reduced by DMTU. ROS, originating from the plasma-membrane NADPH oxidase, which transfers electrons from cytoplasmic NADPH to O_2 to form $O_2^{-\bullet}$, followed by dismutation of $O_2^{-\bullet}$ to H_2O_2, has been a recent focus in ROS signaling. There are reports indicating that oxidative burst and the accumulation of H_2O_2 are mediated by the

activation of plasma-membrane NADPH oxidase complex (Ogawa et al., 1997; del Río et al., 1998; Potikha et al., 1999; Pei et al., 2000; Orozco-Cárdenas et al., 2001; Jiang and Zhang, 2002). Some chemical inhibitors of the NADPH oxidase complex found in mammalian neutrophils, such as diphenyleneiodonium chloride (DPI) and imidazole (IMD), inhibit the pathogen-, elicitor-, wound-, and ABA-induced accumulation of H_2O_2 in plants (Levine et al., 1994; Auh and Murphy, 1995; Bestwick et al., 1997; Alvarez et al., 1998; Orozco-Cárdenas and Ryan, 1999; Jiang and Zhang, 2002). Previously, we also demonstrated that ABA-induced H_2O_2 accumulation in rice leaves can be inhibited by low-concentration (25 μmol L^{-1}) DPI and 0.1 mmol L^{-1} IMD, indicating that ABA-dependent H_2O_2 generation originated, at least in part, from plasma-membrane NADPH oxidase (Hung and Kao, 2004). As shown in Figure 2, when rice leaves were treated with DPI and IMD, ABA-induced accumulation of anthocyanins was reduced.

Effect of phosphatidylinositol 3-kinase (PI3K) inhibitors

The mechanism of ROS production and the molecules involved have been well investigated in animal cells, particularly in neutrophils. The NADPH oxidase complex, which consist of many components, is responsible for ROS production in neutrophil cells, and is activated by the binding of phosphatidylinositol 3-phosphate to one of the components (Ellson et al., 2001). Phosphatidylinositol 3-phosphate is a product of PI3K. Jung et al. (2002) and Park et al. (2003) demonstrated that wortmannin (WM) or LY 294002 (LY), inhibitors of PI3K, inhibited ABA-induced H_2O_2 production and stomatal closing and H_2O_2 partially reversed the effects of WM or LY on ABA-induced stomatal closing. They suggested that phosphatidylinositol 3-phosphate is important in NADPH oxidase-mediated H_2O_2 production during ABA-induced stomatal closing. In our previous work, we showed that WM or LY prevented ABA-induced H_2O_2 production (Hung and Kao, 2005b). In the present study, we demonstrated that ABA-induced anthocyanin accumulation in rice leaves was reduced by WM or LY (Figure 3). Meanwhile, we also observed that H_2O_2 reversed the effect of WM or LY on ABA-induced increased anthocyanin content (Figure 3).

Effect of nitric oxide donor

Nitric oxide (NO) is a bioactive free radical implicated in a number of physiological processes

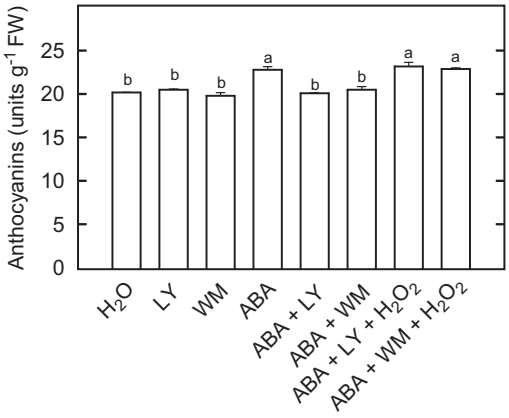

Figure 3. Effect of WM or LY on anthocyanin content in ABA-treated rice leaves in the presence or absence of H_2O_2. The concentrations of ABA, WM, and H_2O_2 were 45, 1, 100 μmol L^{-1}, and 1 mmol L^{-1}, respectively. All measurements were determined 2 d after treatment in the dark. Vertical bars represent standard errors ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

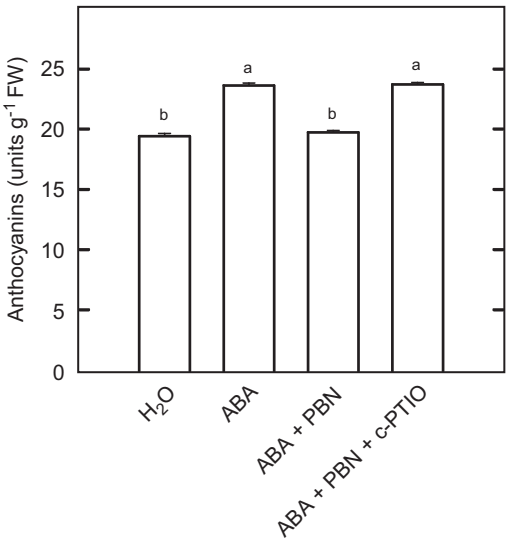

Figure 4. Effect of PBN on anthocyanin content in ABA-treated rice leaves in the presence or absence of c-PTIO. The concentrations of ABA, PBN, and c-PTIO were 45, 100, and 100 μmol L^{-1}, respectively. All measurements were determined 2 d after treatment in the dark. Vertical bars represent standard errors ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

in plants, including growth, development, and defense responses (Lamattina et al., 2003). It has been shown that NO is able to counteract the toxicity of paraquat and diquat, which are known to generate superoxide radicals, in potato and rice leaves (Beligni and Lamattina, 1999; Hung et al., 2002). More recently, we have shown that

ABA-induced H_2O_2 production in rice leaves can be reduced by the NO donor *N-tert*-butyl-α-phenylnitrone (PBN) (Hung and Kao, 2003). Here, we show that PBN is effective in reducing ABA-induced accumulation of anthocyanins in rice leaves (Figure 4). Meanwhile, these PBN effects can be reversed by 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO), an NO-specific scavenger (Figure 4), suggesting that the PBN effects are attributable to NO release.

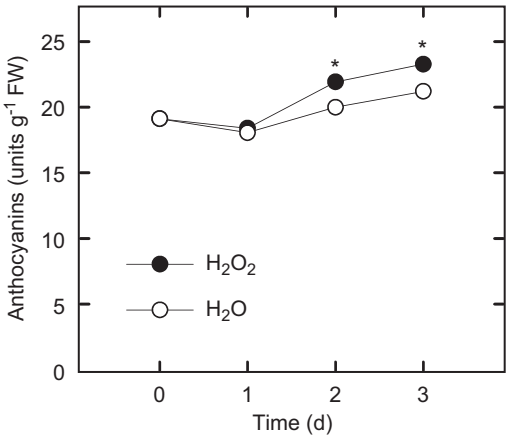

Figure 5. Changes in anthocyanin content in rice leaves treated with either water or 10 mmol L^{-1} H_2O_2 in the dark. Vertical bars represent standard errors ($n = 4$). Asterisks represent values that are significant at $P < 0.05$ by Student's *t*-test when compared with water control.

Effect of exogenous H_2O_2

If H_2O_2 indeed plays an important role in ABA-induced accumulation of anthocyanins in rice leaves, exogenous H_2O_2 is expected to increase the content of anthocyanins in rice leaves. As shown in Figure 5, it is indeed the case.

ABA induces H_2O_2 and anthocyanin accumulations in the leaves of cultivar TN1 seedlings but not in cultivar TNG67

Figure 6 shows the effect of ABA, in the range 5–40 μmol L^{-1}, applied over a period of 3 d, on the contents of H_2O_2 and anthocyanin in the second leaves of rice seedlings. It is clear that increasing ABA concentration progressively increases anthocyanin content in leaves of TN1 seedlings but not in leaves of TNG 67 (Figure 6B and D). It appears that, in terms of anthocyanin accumulation, TNG67 is an ABA-insensitive cultivar and TN1 is an ABA-sensitive cultivar. If H_2O_2 is important in regulating anthocyanin accumulation, then H_2O_2 content is expected to be increased in ABA-treated seedlings

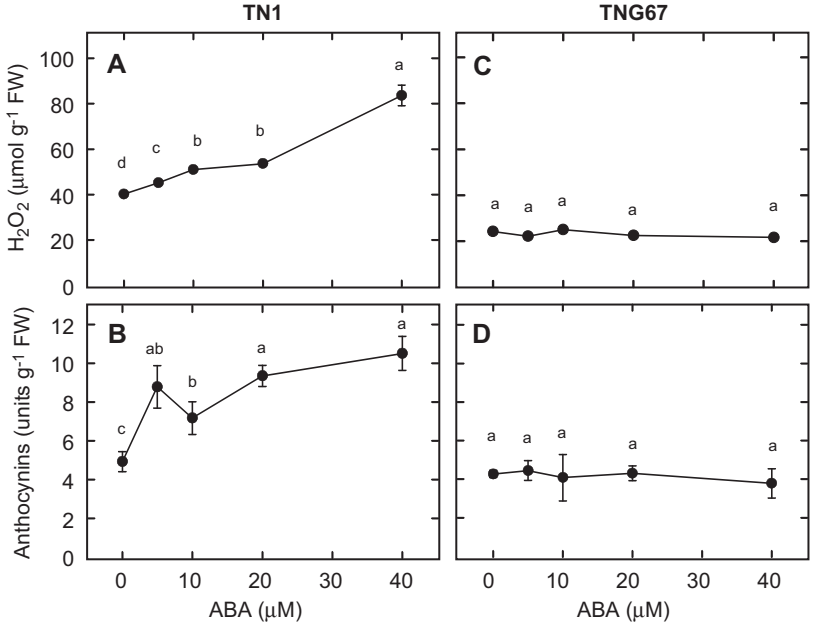

Figure 6. Effect of ABA on the contents of H_2O_2 (A, C) and anthocyanins (B, D) in the second leaves of rice seedlings. Rice seedlings were cultivated in Kimura B solution (half-strength) in a Phytotron with natural sunlight at 30 °C (day)/25 °C (night) at 90% relative humidity. ABA was added to the Kimura B solution when the third leaves were fully expanded. H_2O_2 and anthocyanin content were determined 3 d after adding ABA. Vertical bars represent standard errors ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

of TN1 but not in TNG67. As indicated in Figure 6A and C, this is the case.

Treatment with ABA resulted in a two-fold increase in anthocyanin content in the second leaves of TN1 seedlings (Figure 6B). However, ABA caused only about a 3% increase in anthocyanins (Figure 1). In experiments with intact leaves of TN1 seedlings, ABA was added to culture solution under natural sunlight conditions. However, for detached leaf system, leaf segments were treated with ABA in the dark. It appears that ABA affect on anthocyanin accumulation is more pronounced in the light than in darkness.

Conclusion

It has been shown that the increase in the content of H_2O_2 and the specific activity of PAL, a key enzyme of anthocyanin biosynthesis, occurs 24 and 36 h, respectively, after ABA treatment (Hung and Kao, 2004, 2005a). In the present work, we show that anthocyanin accumulation occurs 48 h after ABA treatment (Figure 1). Clearly, H_2O_2 production is prior to the increase in PAL specific activity and anthocyanin content in ABA-treated detached leaves. In terms of anthocyanins, it was demonstrated that rice seedlings of TN1 are ABA sensitive and those of TNG are ABA insensitive. Upon treatment with ABA, H_2O_2 accumulated in the leaves of TN1 seedlings but not in the leaves of TNG67. This work establishes the links between ABA treatment, H_2O_2, and anthocyanins.

Acknowledgments

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