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Effects of Abscisic acid and Temperature on the Anthocyanin Accumulation in Seedlings of *Arabidopsis thaliana*

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Effects of abscisic acid(ABA) and temperature on the anthocyanin accumulation and phenylalanine ammonia lyase(PAL) activity were investigated in seedlings of *Arabidopsis thaliana*. In time course study, exogenous application of ABA (50-1000 μM) led to a noticeable increase in anthocyanin pigments which persisted over the following 5 days. Anthocyanins increased in concert with the chlorophyll loss. The activity of PAL, a key enzyme in the phenylpropanoid pathway, increased on exposure to ABA and reached maximum on the 4th day. This result shows that anthocyanin synthesis and PAL activity have a close physiological relationships. In the effects of temperatures (10℃, 17℃, 25℃ and 30℃) on anthocyanin accumulation and PAL activity in seedlings, a moderate-low temperatures (17℃) enhanced both anthocyanin content and PAL activity, whereas elevated temperatures (30℃) showed low levels of anthocyanin and PAL activity, suggesting a correlation between temperature-induced anthocyanin synthesis and the accumulation of PAL mRNA. Simultaneous application of ABA with temperatures induced higher anthocyanin synthesis and PAL activity in seedlings than ABA or temperature stress alone. Moderate-low temperature with ABA exposure elicited the maximal induction of anthocyanin synthesis and PAL activity. Therefore, ABA treatment significantly increased thermotolerance in *A. thalinan* seedlings. Ethephon and ABA showed similar mode of action in physiological effects on anthocyanin accumulation and PAL activity. Our data support that anthocyanins may be protective in preventing damage caused by environmental stresses and play an important role in the acquisition of freezing tolerance.

Key Words : Anthocyanin, Abscisic acid, Ethephon, Low temperature, Phenylalanine ammonia lyase(PAL), *Arabidopsis thaliana*

1. Introduction

The characteristic red, violet and blue coloration seen in petals, stems and fruits of higher plants is due to anthocyanins either in part or exclusively. Anthocyanins are water-soluble pigments derived from flavonoids via the shikimic acid pathway[1]. Synthesized in the cytoplasm and actively sequestered into cell vacuole by a glutathione pump. The role of anthocyanins is not clear and may depend on whether their location is in the vacuoles of the abaxial or adaxial leaf epidermis, in the cytosol of mesophyll cell, in roots, or in stems[2].

The visual function of anthocyanins in reproductive organs as an aid to pollination and seed disposal is generally accepted. Other proposed roles for anthocyanins include protection from photoinhibition and the effect of UV-B, defense against hervivores and free radical scavenging[3,4]. However, ascribing a function to the transient accumulation of anthocyanin in green, vegetative tissues has proven elusive. Anthocyanins may be developmentally transient, appearing only in juvenile or senescing tissues, or they may be permanent. Many herbaceous seedlings accumulate anthocyanins transiently, often as a result of photoinduction and within hours or days after germination[5]. Seedlings of woody species seem to require additional environmental cues such as cold temperature to accumulate anthocyanins, while their mature counterparts do not demonstrate this ability.

The induction of anthocyanins by environmental

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stresses, the appearance of red leaves at predictable times of the year and at specific stages in leaf development, and their prominence in particular environmental niches have promoted many workers to postulate roles for anthocyanins in leaves. However, the literature on their environmental induction and subsequent impact on plant survival, especially in regard to nonreproductive tissues has not been reviewed. In terms of adaptive advantage, leaf anthocyanins may be the least understood group of nonphotosynthetic pigments in plants.

The synthesis of anthocyanins is affected by various environmental factors, such as light, temperature, nutrition, infection and drought. Anthocyanins in leaf tissues have a dual function as absorbers of harmful levels and/or wavelengths of radiation and as osmotic adjusters[6]. Krol et al.[7] speculate that the phenomenon of anthocyanin development in young *Pinus* seedlings may somehow help them establish under a suite of suboptimal environmental conditions including photooxidation, low temperature, water and nutrient stress. Temperature is one of the main external factors affecting anthocyanin accumulation in plant tissues. Suboptimal temperatures, experienced either as sudden, short-term cold spells or long-term seasonal reductions in temperature, induce anthocyanin synthesis[8], while elevated temperatures reduce anthocyanin synthesis and are associated with net pigment loss[9]. Furthermore, in several plant systems, such as maize seedlings[10], *Arabidopsis*[1] and petunia[11] the temperature has been to have a significant effect on the expression of anthocyanin genes.

Anthocyanins disappear with the resumption of growth with increasing temperature, although it may persist with the continuation of cold conditions[9]. Conversely, anthocyanin synthesis may coincide with resumption of photosynthetic activity and increasing temperatures in cold environments. Environmental and growth conditions that predispose the photosynthetic apparatus to photoinhibition and photooxidation may increase the extent of anthocyanin accumulation in response to low temperature[12].

The induction of anthocyanins by chilling temperatures does suggest a protective function, and some studies are supportive of this idea. McKown et al.[13] suggest some commonality between anthocyanin biosynthesis and freezing tolerance, as four *Arabidopsis* mutants deficient in freezing tolerance were unable to accumulate anthocyanins. Anthocyanin-rich species such a *Photinia* have extended growing petioles compared to other ornamental shrubs, perhaps as a result of increased tolerance of cool temperatures.

Anthocyanins, which accumulate in leaves and stems in response to low temperature and changes in light intensity, are synthesized through the phenylpropanoid pathway that is controlled by key enzymes that include plenylalanine ammonia lyase(PAL) and chalcone synthase(CHS). Leyer et al.[1] demonstrated that PAL and CHS mRNAs accumulate in leaves of *Arabidopsis thaliana* upon exposure to low temperature in a light-dependent manner. PAL activity has been shown to increase in response to low temperature in different species[15], and the accumulation of PAL protein during cold acclimation has been demonstrated in *Brassica napus*[16]. Furthermore, a correlation between low temperatures induced anthocyanin synthesis and the accumulation of PAL and CHS mRNA has been demonstrated in maize[10]. It should be note, however, that low temperatures in the absence of either visible light or UV-B prevent anthocyanin biosynthesis. As Mol et al.[17] conclude, the mechanism of cold induction of anthocyanins and the role of light are not fully understood and they again suggest separate, or perhaps overlapping pathways.

The impact of exogenous growth regulators on anthocyanin accumulation is also unclear. Both auxin and/or cytokinins have been shown to induce anthocyanins in cell cultures[18] or whole plant systems[19], and others have linked gibberellins to anthocyanin production[17]. However, Ronchi et al.[20] concluded that anthocyanin accumulation in *Zea mays* was instead linked to gibberellin inhibition. Likewise, gibberellins were seem to have no effect on anthocyanins in *Photinia* leaves. Fambrini et al.[21] suggest that lack of abscisic acid prevents anthocyanin manufacture, although others have noted an inhibitory effect of abscisic acid[17].

In seedlings of *Arabidopsis thaliana*, we found that the pattern of anthocyanin accumulation was influenced by the temperature during irradiation. Exogenous application of abscisic acid frequently leads to a measure of protection against a variety of environmental stresses, such as chilling, freezing, drought and

salinity. Abscisic acid has been proposed as a necessary mediator in triggering of many physiological and molecular adaptive responses to adverse environmental conditions and abscisic acid-induced stress tolerance is often associated with abscisic acid-regulated gene expression[22]. There has been considerable interest in the role of abscisic acid in mediating tolerance to low temperature and heat stress. The aim of the present study was to determine the involvement of abscisic acid and temperature in the induction of anthocyanin accumulation in plants using a combination of different approaches. The changes in anthocyanin accumulation, chlorophyll content and PAL activity were compared for exogenous abscisic acid affected seedlings of *Arabidopsis thaliana* exposed to different temperatures.

2. Materials and Methods

2.1. Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* (L.) Heinh. (Columbia ecotype) were surface-sterilized in 10% sodium hyperchloride for 5 min and washed completely with sterile water. Seeds were sown on wet filter paper in Petri dishes and incubated for 2 days in darkness at 4℃. Seedlings were then planted in pots containing a mixture of perlite and vermiculite and were irrigated with MS solution.

Plants were grown in a growth cabinet(Eyelatron FLI-301N, Japan) at a day / night temperature of 22 / 18℃, a light regime of 100 μmol $m^{-2}s^{-1}$, provided by cool-white fluorescent lamps with a 16 h photoperiod and 70 % relative humidity.

Five-day-old seedlings were exogenously applied to either abscisic acid or ethephon for 5 days at the following concentrations : 0(control), 50, 100, 250, 500 and 1000 μM.

For time-course analysis, the seedlings were harvested at different time intervals for the estimation of anthocyanins or phenylalanine ammonia lyase activity. After being exposed to abscisic acid and ethephon for 5 days, individual seedlings were harvested for assay.

For the temperature treatments, plants grown in the growth cabinet were divided into four pots of 10 plants, respectively. Four day / night temperature regimes were selected : 10℃/5℃, 17℃/12℃, 25℃/20℃ and 30℃/25℃, all at 16 h light and 8 h dark photoperiod.

Each experiment was repeated three times independently and the mean and SE values were determined. Abscisic acid or ethephon was applied alone or simultaneously with temperature to the intact seedlings.

2.2. Estimation of anthocyanins

Anthocyanins were extracted by submerging the organs in acidified 70% methanol (CH_3OH) that contained 1% HCl at 4°C for 24 h. The extract was filtered through Whatman #4 filter paper, and anthocyanin content was estimated by measuring absorbance at 535 nm with UV/VIS spectrophotometer[23].

2.3. Estimation of chlorophyll

Each preweighed leaf was homogenized with a mortar and pestle in 80%(v/v) acetone and contrifuged at 10,000×g for 10 min at 0-4℃. The supernatant was brough up to a 1 ml volume, then the absorbance of the acetone extract was measured at 663 and 645 nm using a UV/VIS spectrophotometer. The chlorophyll content was culculated according to the method of Arnon[24].

2.4. Phenylalanine ammonia lyase(PAL) assay

The leaf segments were homogenized at 4℃ in a pre-cooled motar and pestle with 200 mg sea sand in 10 ml of 25 mM borate buffer(pH 8.8) containing 3 mM 2-mercaptoethanol. The homogenate was centrifuged at 18,000×g for 20 min at 4℃ and the supernatants were used for assay.

The PAL assay was performed at 40℃ for 2 h in an assay mixture consisting of 0.5 ml of enzyme extract and 2.5 ml of 10 mM L-phenylalanine. The reaction terminated with 0.1 ml of 5 N HCl. The PAL activity was assayed by monitoring the increase in A_{290} against a control without phenylalanine over a period of 4 h at 1 h intervals. The rate of appearance of *trans*-cinnammic acid was taken as a measure of enzyme activity[25]. The PAL activity is expressed in pkat(pmol *trans*-cinnammic acid formed s^{-1}) g^{-1} of tissue.

3. Results and discussion

3.1. Effect of ABA and ethephon on anthocyanin accumulation

The application of abscisic acid (ABA) at concen-

trations from 50 to 1000 μmol to *A. thaliana* seedlings stimulated anthocyanin production. The visual symptoms by ABA treatment ranged from a slight reduction in growth to complete violet coloration of the entire seedling, depending on the extent of treatment(Fig. 1). Seedlings which had developed on ABA-treated plants showed smaller shoot, shortened petiole and reduced leaf surface than those from control plants.

The time-course of anthocyanin induction by ABA treatment was compared in different concentrations of ABA in *A. thaliana* seedlings after a 5 day of exposure. Fig. 2 shows that in the above seedlings the increase in anthocyanin level continued up to 5 day. The amount of anthocyanin increased as the incubation time was increased and the concentration of ABA was higher. The onset of anthocyanin was hastened depending on the concentration of ABA. In seedlings exposed to 500 μM ABA a slow rise was observed in anthocyanin level up to 4 day, followed by a rapid increase in anthocyanin level and the maximal rise was recorded at the end of experiment. In contrast, the anthocyanin accumulation in the control plants exhibited a small fluctuating pattern during the seedling growth. The increase in the anthocyanin content in the seedlings was nearly 2.5-fold in comparison to that in control plants. Ethephon treatment was also found to be most effective in inducing anthocyanin production in organs(Fig. 3). Higher concentration of ethephon induced maximal production of anthocyanin.

Time course for the chlorophyll behavior in seedlings as a function of ABA is shown in Fig. 4. A significant decrease in the chlorophyll level was observed in the ABA-treated leaves. Two compounds had the same effect on the anthocyanin and chlorophyll synthesis in seedlings of *A. thaliana* (Table 1). 500 and 1000 μM ABA treatment induced an inhibition of 70 and 78 % in total chlorophyll yield, while 500 and 1000 μM ethephon treatment induced an inhibition of 83 and 94 %, respectively. Therefore, the promotion of chlorophyll loss was concentration-dependent under ABA and ethephon treatment. Anthocyanins increased in concert with the chlorophyll content. AC levels, which varied 3.5-fold across the sample as a whole, correlate strongly with levels of chlorophyll in the leaves.

The strong association of anthocyanins with chlorophyllous cells indicates a primary role in photosynthesis, perhaps by protecting chloroplasts from photoinhibition during periods of high photon flux[26]. Numerous workers have reported a reduction in photosynthic efficiency associated with anthocyanin pro-

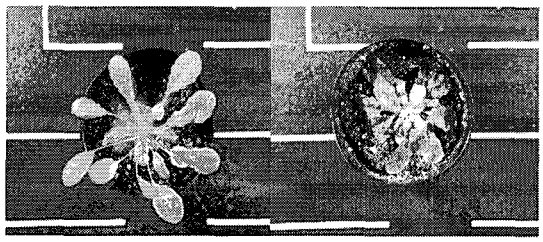

Fig. 1. Seedlings of *Arabidopsis thaliana* grown for 5 days in nutrient solution in the absence(left) or presence(right) of 500 μM ABA.

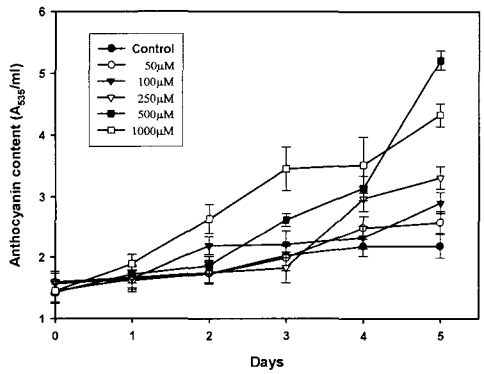

Fig. 2. Time course of the accumulation of anthocyanin in *Arabidopsis thaliana* seedlings treated with different concentrations of ABA for 5 days.

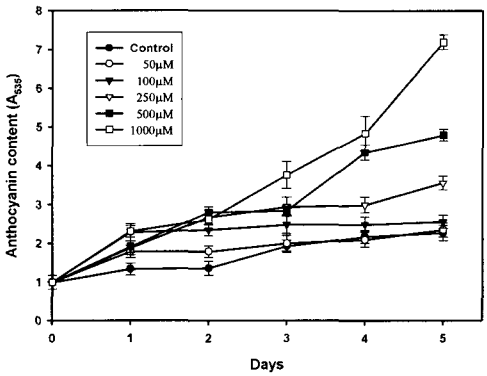

Fig. 3. Time course of the accumulation of anthocyanin in *Arabidopsis thaliana* seedlings treated with different concentrations of ethephon for 5 days.

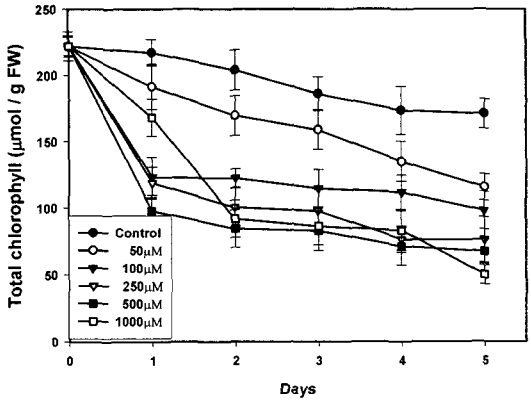

Fig. 4. Time course of the chlorophyll content in *Arabidopsis thaliana* seedlings treated with different concentrations of ABA for 5 days.

Table 1. Effect of ABA and ethephon treatment on the anthocyanin and chlorophyll synthesis in seedlings of *Arabidopsis thaliana*

Treatment		Anthocyanin content (A_{535} / g FW)	Chlorophyll content (μmol / g FW)
ABA	0 μM	1.2 ± 0.27	220 ± 0.7
	500 μM	5.2 ± 0.92	67 ± 0.8
	1000 μM	4.2 ± 0.30	49 ± 0.2
Ethephon	0 μM	1.2 ± 0.08	220 ± 0.8
	500 μM	4.2 ± 0.27	37 ± 0.3
	1000 μM	7.1 ± 0.14	13 ± 0.2

duction[3,27], although the mechanism by which this is achieved remains obscure. However, one postulate that the absorption of UV-A / blue light by anthocyanins reduces chlorophyll and carotenoid synthesis, clearly dose not apply to *Quintinia serrata*[28]. Anthocyanin in *Q. serrata* may serve to protect shade-adapted chloroplasts from brief exposure to high intensity sunflecks.

ABA have been implicated in promoting senescence by affecting chlorophyll levels, probably by disruption of several processes associated with chlorophyll synthesis and destruction. Increased ABA levels accelerate, in the long term, ripening of fruits and lead to prematuration of leaf and fruit drop. ABA had a profound effect on anthocyanin production in terms of anthocyanin content which supports the results of some studies[21]. Overall production of anthocyanin was much higher in ABA treatments. A recent paper could conciliate these conflicting results. There was an evidence that lack of ABA prevents anthocyanin manufacture. Above all, it is shown that the stimulatory effect of ABA on anthocyanin accumulation is dependent on light mediated processes. The mechanism undelying the cooperative effect of light and ABA also remains to be elucidated.

Some environmental stresses have been well known to produce ethylene[29] and the application of the ethylene-generating compound ethephon induces leaf and flower senescence, fruit ripening and abscission of organs[30]. Anthocyanin accumulation is regulated by ABA and its related compounds in a way similar to the mode of action of ethylene. It has been shown that ABA interact with ethylene biosynthesis and ABA and ethylene frequently show similar biological effects.

In order to characterize the effect of ABA on pigmentation, activities of enzyme involved in the anthocyanin biosynthetic pathway, phenylalanine ammonia lyase(PAL) were measured in seedlings exposured to 500 μM ABA for 5 days (Fig. 5). There was a significant difference between the ABA-treated and control plants. PAL activity began to increase from the first day, reaching maximum level on the 4th day and declining by the 5th day of exposure. Similarly, in ethephon-treated seedlings, a gradual rise

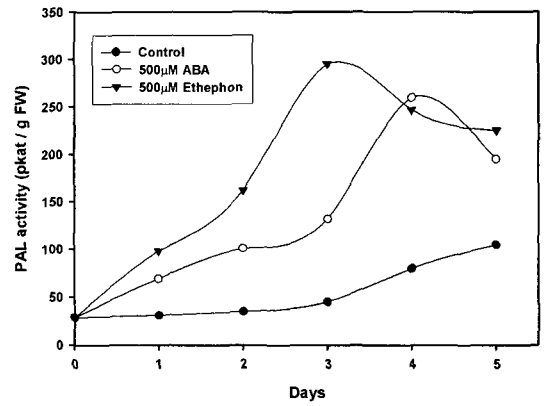

Fig. 5. Time course of PAL activity in *Arabidopsis thaliana* seedlings treated with 500 μM ABA and ethephon for 5 days.

in PAL activity was observed up to 3th day, followed by a maximum rise in the activity. Although the seedlings makes a gradual rise of anthocyanin, it is plausible that the actual peak of PAL may be between 3-4 day in all organs.

The photoinduced accumulation of flavonoids is preceded by the induction of several enzymes involved in phenylpropanoid metabolism[31]. Photoregulation of enzymes involved in anthocynin and other flavonoids biosynthesis including PAL and CHS have been studied in detail in many systems[32]. It is plausible that photoreceptors such as UV-B induced PAL enzyme may play a role in anthocyanin formation in maize seedling[33].

Very little is known about the catabolism of anthocyanins in plant tissues. Anthocyanin degradation was followed in these studies by inhibiting PAL activity, thus preventing anthocyanin synthesis. The rate of degradation varied with tissue maturity and with environmental conditions. Nitrogen deficiency, low temperature stress and application of growth retardants have been correlated with increased anthocyanin synthesis in plant tissue[34].

3.2. Effect of temperature on anthocyanin accumulation

In order to determine if the increase in anthocyanin concentration was directly correlated to the increase in temperature, 5-day-old seedlings were grown for 5 days in the growth cabinet at high (30/25℃), moderate (25/20℃), moderate-low (17/12℃) and low (10/5℃) temperatures. Seedlings which had developed at the high temperatures caused fading of leaf colour and had a low level of anthocyanin, whereas those grown at moderate-low temperatures had higher levels of anthocyanin than those from the high temperature treatment (Fig. 6). The anthocyanin concentration of seedlings grown at 17℃ was about 3.5-fold higher than that of seedlings grown at 30℃.

Environmental factors or stresses including mechanical wounding, fungal elicitors and temperature affect anthocyanin synthesis in various plant tissues[10]. Seedlings of *A. thaliana* that develop at moderate-low temperature (17℃) contain more pigment than those grown at high temperatures. Moderate-low temperatures(10-15℃) enhances anthocyanin synthesis in sorghum, cabbage and maize seedlings[10,35]. Low temperatures has previously been shown to act as a stress signal that increase anthocyanin synthesis. In *A. thaliana* leaves, pigment accumulation in response to low temperature results from the activation of PAL and CHS gene transcription in a light-dependent manner[1]. The increasing levels of anthocyanin in apple skin coincide with decreasing temperature[36]. In roses, a rise in temperature results in a lower concentration of anthocyanins, whereas cooling rose buds enhances both weight and pigmentation[37].

The site of anthocyanin accumulation was influenced by temperature during irradiation in *Polygonum cuspidatum* seedling[38]. Anthocyanin accumulated first in the lower part of hypocotyls and then towards the upper part of hypocotyls of seedlings irradiated with white light (WL) at 25℃, whereas anthocyanin accumulated only in the upper parts of etiolated seedlings irradiated with WL at 5℃. Spectral sensitivity was also dependent on the temperature during irradiation. Red light, blue light and near ultra-violet (NUV) light induced the accumulation of anthocyanin at 5℃ but only NUV was effective in inducing the accumulation of anthocyanin at 25℃.

Simultaneous application of ABA with temperature strongly enhanced the synthesis of anthocyanin as compared with the treatment of ABA or temperature alone (Fig. 6). Anthocyanin levels were almost 4-fold higher in seedlings treated with 500 μM ABA at moderate-low temperature (17℃) than those treated ABA at high temperature (30℃). Seedlings grown under four temperature regimes and treated with ethephon showed a similar response to temperature to that found in ABA treatment (Fig. 7). The application of 500 μM ethephon to seedlings at 17℃ induced almost 3.5-fold higher anthocyanin level in comparison to that at 30℃. It has been reported that ABA plays an important role in mediating plant responses to environmental stresses. ABA significantly increases cold, drought, wounding, salt resistance, anoxia tolerance and freezing tolerance in several plant species[39,40].

The role of ABA in low temperature stress was deduced from various findings, including an increase in endogenous content during low temperature treatment, the increase of plant freezing tolerance induced by ABA application under nonacclimation conditions, and reduced tolerance to freezing in ABA-mutant plants. ABA treatment significantly increased thermotolerance in maize seedlings[39]. The ABA-induced thermotol-

erance is mediated by calcium and associated with ABA-induced increase in the activities of antioxidant enzymes. The effect of exogenous ABA application on thermotolerance varies depending on plant materials. Bray[41] reported that ABA treatment significantly reduced the thermotolerance of wild-type tomato leaves. On the other hand, ABA treatment was shown to improve the recovery growth of maize seedlings after heat treatment[42] and to enhance the survivial percentage of bromegrass cell suspension cultures at 42.5℃ [43]. ABA, therefore, has been proposed as a necessary mediator in many physiological and molecular adaptive responses to adverse environmental conditions, and ABA-induced stress tolerance is often associated with ABA-regulated gene expression[22,44].

In order to characterize the effect of temperature on pigmentation, PAL activities were measured in seedlings grown under the four temperature regimes (Fig. 8). Seedlings grown at 30℃ had a low level of PAL activity, whereas those grown at moderate-low temperature(17℃) had higher levels of PAL than those from high temperature treatment. PAL analysis of seedlings exposure to 17℃ revealed a 2.5-fold higher PAL level. The effect of 500 μM ABA and temperature, administered in combination, on PAL activities were determined in 10-day-old seedlings (Fig. 8). ABA treatment was more effective in inducing PAL activity in leaves, and moderate-low temperature with ABA exposure elicited the maximal induction of PAL activity in *A. thaliana* seedlings. By contrast, the seedling exposed to ABA at high temperature showed much lower induction of PAL activity. Ethephon treatment was also found to be most effective in inducing PAL synthesis in leaves, though PAL levels were lower than those from ABA treated plants (Fig. 9).

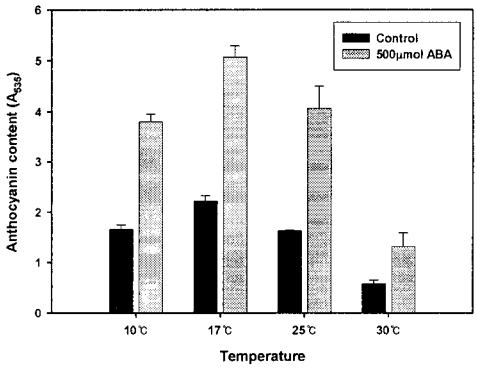

Fig. 6. Anthocyanin contents in seedlings of *Arabidopsis thaliana* exposed to different temperatures and 500 μM ABA alone and in combination.

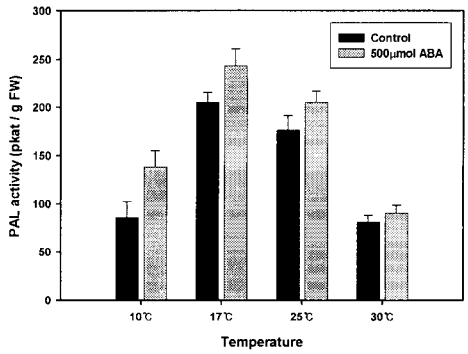

Fig. 8. PAL activities in seedlings of *Arabidopsis thaliana* exposed to different temperatures and 500 μM ABA alone and in combination.

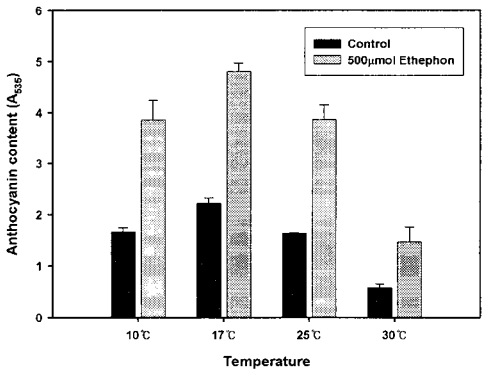

Fig. 7. Anthocyanin contents in seedlings of *Arabidopsis thaliana* exposed to different temperatures and 500 μM ethephon alone and in combination.

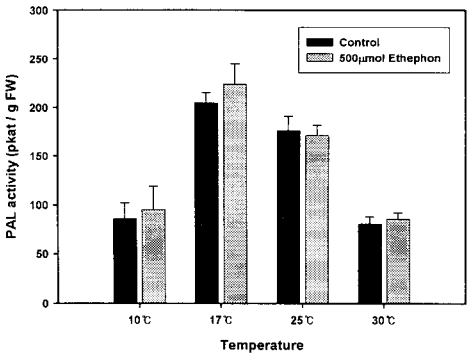

Fig. 9. PAL activities in seedlings of *Arabidopsis thaliana* exposed to different temperatures and 500 μM ethephon alone and in combination.

PAL activity has been shown to increase in respose to low temperature in different species[15], and the accumulation of PAL protein during cold acclimation has been demonstrated in *Brassica napus*[16]. Furthermore, a correlation between low temperature- induced anthocyanin synthesis and the accumulation of PAL mRNA has recently been demonstrated in maize[10].

Here we have shown that the activity of PAL, a key enzyme in the phenylpropanoid pathway, decreased in *A. thaliana* seedlings in response to elevated growth temperatures. PAL activity remarkably decreased at 30℃, but at 17℃ significant change in activity was detected during seedling growth. This suggests that PAL activity is under different control than enzymes solely committed to flavnoid and anthocyanin biosynthesis. Most research on temperatures and anthocyanins has been focused on metabolic pathways and environmental factors causing increased synthesis. However, the effect of elevated temperatures on anthocyanin stability has little investigated. It is possible that the stability of anthocyanin in living tissues decreases at high temperatures.

The present study shows that *A. thaliana* seedlings on exposure to ABA and/or low temperatures accumulate a higher anthocyanin level, which may perhaps be defence response against high temperature stress, suggesting that anthocyanin production is predomiant mechanism in the young plants and anthocyanin function as photoprotective pigments. From the above results, it is suggested that anthocyanin production is predominant mechanism of protection in the young plant and anthocyanins play an important role in the acquisition of freezing tolerance. Further investigations on the causes of damage due to UV radiation, freezing, wounding and pathogen infection at different temperatures, and the real effect of stress hormones on the anthocyanin synthesis will be interest. More work is needed to examine the defence response of anthocyanins against environmental stresses.

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