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TOBACCO EXPRESSING *PAP1* INCREASES THE RESPONSES TO PAR AND UV-A BY ENHANCING SOLUBLE SUGARS AND FLAVONOIDS AND ELEVATING PLANT PROTECTIONS

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Abstract

Five lines of transgenic tobacco over-expressing Production of Anthocyanin Pigment 1 (*PAP1*) cDNA were analysis of metabolic response against the radiation and their protection of the plant under tissue culture condition. *PAP1* transgenic and wild type (WT) plants were treated with the radiations of photosynthetically activate radiation (PAR) or PAR combined with UV-A. All lines of transgenic significantly increased in amounts of *p*-coumaric acid, naringenin apigenin more than WT under both treatments. Additional UV-A radiating to plant rose up kaempferol content in WT plant (1.5 times) and in *PAP1* transgenics (1.8 times). These transgenic plants treated under both conditions had also increased anthocyanin substances (pelargonidin) with significant value after compared to WT. Content of total soluble sugar (TSS) was related to the content of total flavonoids in transgenic. PAR combined with UV-A had a lower induction of the electrolyte leakage percentage and malondialdehyde (MDA) level in the transgenic leaf tissue compared to WT tissue. The metabolic substance levels were considered on its protection of plant cells. In transgenic tissue, the enhancement of apigenin level strongly diminished the increase level of electrolyte leakage while the levels of TSS, *p*-coumaric acid and naringinin less affected. Moreover, the increase levels of kaempferol and pelargonidin associated with the decrease level of MDA, while the TSS level reversely responded. *The PAP1 transgenic increased response* of light by adaptation of their metabolites (TSS, *p*-coumaric acid and flavonoids) consequently enhance parameter indicating protections of the cell.

Key words: Plant protection; Flavonoids; Lights; *PAP1*; Soluble sugar.

Introduction

The energy source of light has a major function in electron transfer of the plant photosynthetic process. The suitable wavelength of light for photosynthetic process is 400-700 nm or photosynthetically activating radiation (PAR). A wavelength of ultraviolet (UV) light is a high-electromagnetic energy, therefore their radiation has prospective to case a damage plant physiology. Among UV light, long wavelength of UV-A (315-400 nm) have the least detrimental effect to plant. UV radiating to plant cell affected an enhancement of reactive oxygen species (ROSs). A high accumulation of ROSs consequently induced the organelle and cell damages (Blokhina *et al*., 2003). Plants have evolved several mechanisms against stresses in order to decrease cell damage. Individual plant may differ in the protecting system.

Carbohydrate quality and quantity in the plant has been reported that related to stress responses (Maness, 2010). Soluble carbohydrates have a basal role as energy sources, osmolytic agents and precursors. These precursors convert into other useful substances. Secondary metabolites from phenylpropanoids pathway accumulated in the UV radiated epidermal cells have been reported (Bruns *et al*., 1986; Christie & Jenkins, 1996; Wade *et al*., 2001). These metabolites producing in plant cell have been found to screen an unappropriated radiation so as to reduce cell damages (Robberecht & Caldwell, 1978; Burchard *et al*., 2000; Landry *et al*., 1995). Plants mutated genes in the biosynthesis of phenylpropanoids and flavonoids were vulnerable to the radiation of UV (Stapleton & Walbot, 1994; Landry *et al*., 1995).

Different light signals case the activation of cascade proteins and metabolic biosynthesis a wide range of plant. The MYB protein, a plant transcription factor, plays a critical role in controlling gene expression, including in the flavonoid pathway (Jin & Martin, 1999). The *production of anthocyanin pigment 1 (PAP1)* gene was isolated from *Arabidopsis thaliana*. This gene contains R2R3MYB conserve domain that has been proven to regulate the biosynthesis of phenylpropanoid (Borevitz *et al*., 2000). Over-expressing of *PAP1* activated the expression of genes in phenylpropanoid biosynthesis of whole plants (Borevitz *et al*., 2000; Tohge *et al*., 2005). PAP1 protein has been reported that formed a complex with WD-repeat and basic helix-loop-helix (bHLH) proteins and activated late biosynthesis of the flavonoid pathway (Zhang *et al*., 2003) (Fig. 1). This protein had an intermediate function in responses to several environmental factors and affected the level of flavonoid biosynthesis in plant (Cominelli *et al*., 2008; Teng *et al*., 2005; Lillo *et al*., 2008). In nature, flavonoid biosynthesis and accumulation in plants is a variable depending on the environmental conditions and developmental stage (Chalker-Scott, 1990). Until present, it is not yet clear whether *PAP1* transgenic distinguish from non-transgenic in producing of metabolic profiles and their response against light signals.

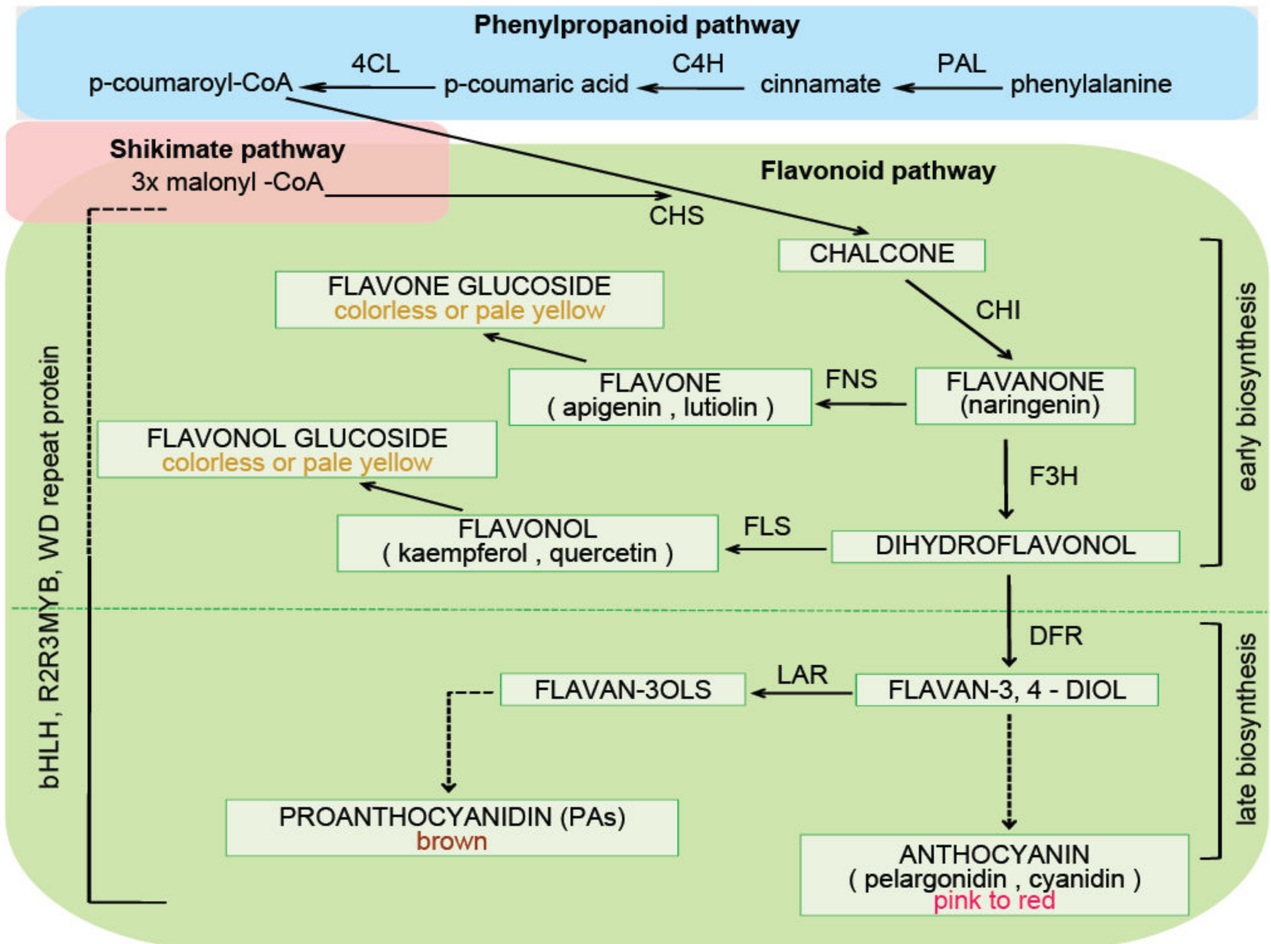

Fig. 1. A schematic represent the early and late of flavonoid biosynthetic genes in the pathway. Enzymes are indicated in bold lettering. CHS is the first enzyme regarding flavonoid production, leading to synthesis of the major flavonoid groups as FLAVANONE, FLAVONE, FLAVONOL, and ANTHOCYANIN showed in white box. Abbreviations are as follows: PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate: CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; FNS, flavone synthase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol reductase; LAR, leucoanthocyanidin reductase. The small letters was showed the derivative of pathways. The arrows indicated step towards major flavonoid derivative production, and dash arrows showed multi-step of enzymes to produce anthocyanins and anthocyanidins (PAs).

In this research, independent lines of *PAP1* transgenic and wild types were used for investigating light responses under tissue culture condition to diminish the environmental change effects. In this experiment, PAR intermixed with UV-A was used for the normal photosynthetic process and mild stimulus which plant physiology had their capacity to retain with. We investigated the accumulating levels of sugar, *p*-coumaric acid and flavonoid substances in *PAP1* transgenic under PAR and PAR+UV-A conditions and compared to those in WT. Moreover, enhancing the level of each substance related to cell protection was analyzed.

Materials and Methods

Plant materials and *In vitro* conditions: Transgenic tobacco with *PAP1* cDNA (accession no. AT1G56650) were constructed according to Kanthang & Sompornpailin methods (2013). Five lines of *PAP1* transgenic containing different levels of substances (line no. P1, P2, P4, P5, P7) and non-transgenic (wild type; WT) were micro-propagated. Plant culture was performed on MS medium (Murashige & Skoog, 1962) under aseptic condition. Propagated lines were used as the sample replicates in experimental studies. The light regime of plant culture condition was 16 h/8 h at 60 μmolm^{-2}s^{-1} of daylight fluorescence tubes (Sylvania Watt/255). This tube provided PAR (400-700 nm). The temperature of the culture room was 25±2°C. Plants were used for 2 conditions of light treatments. Plants were radiated for one week with the combination of PAR and UVA (PAR +UV-A) compared to those with PAR only (control condition). Sylvania black light tubes were used to radiate UV-A (wavelengths between 350-370 nm). Phenotypic changes of each transgenic line under aseptic condition were investigated. Leaves of WT and transgenic plant were collected for analysis, or frozen with liquid nitrogen and kept at -80ºC for further investigation.

Determination of total soluble sugar contents: Zero point five grams of leaf fresh weight (FW) were ground into a powder and extracted with 2 ml of 80% ethanol. Four hundred microliters of supernatant were mixed and extracted with an equal volume of de-ionized water and chloroform. The supernatant was diluted 10 times with deionized water. Five hundred microliters of supernatant dilution was estimated by the phenol-sulfuric acid method of Dubois *et al.* (1956). The absorbance was quantified with a spectrophotometer at 490 nm. Total soluble sugar (TSS) content was calculated from a standard calibration curve of sucrose. Four samples were used in each treatment. The mean in mg of TSS per g FW with error bar in each treatment was presented.

Analysis of flavonoid accumulations: Frozen samples of leaves (1 g FW) were finely pulverized and extracted with 2 ml water and acidic methanol (2:3) solvent. Chlorophyll was separated from solvent by extracting with chloroform. The upper solvent was separated and quantify for specific absorbance using a spectrophotometer. Polyphenol (*p*-coumaric acid) and flavonoid derivatives were analyzed according to the modified methods of Barthelmebs *et al.* (2000) and Harborne, (1998). The absorbance values of substances in each extract were adjusted to the g FW.

Investigation of cell membrane injury by electrolyte leakage: Leaves of tobacco grown under PAR+UV-A were cut into pieces. De-ionized water was added to each leave sample. The UV-A treated samples were incubated at 30ºC for 3 hours. Subsequently, an initial electrical conductivity (EC_i) of the treated samples was measured using a conductivity meter. The sample was heated for 2 min at 100ºC and cooled down with ice. A final electrical conductivity (EC_f) of this sample was measured again. The total electrical conductivity of normal samples (plants grown under PAR) at the initial and final stages (EC_{Ti}, EC_{Tf}, respectively) was measured in the same way as the treated sample. The percentages of electrolyte leakage under PAR or additional UV-A conditions were calculated by following the method of Bajji *et al.* (2002).

Investigation of lipid peroxidation assay: The lipid peroxidation level was determined in plant cell grown under PAR and additional UVA. The thiobarbituric acid-reactive-substances (TBARS) assay was performed according to the method of Hodges *et al.* (1999). Thiobarbituric acid (TBA) is a reactive substance with malondialdehyde (MDA) to yield a pink colored product of TBARS. A fresh leaf sample (0.15 g) was carried out the MDA extraction with 1.5 ml of 1% trichloroacetic acid by shaking for 1 hour at room temperature. Insoluble residue was removed by centrifugation. The supernatant was diluted and added to the TBA solution. The reaction was well mixed and boiled in water for 30 min and then cooled down. The TBARS in the reaction was quantified by spectrophotometry at 532 nm against a blank. The MDA equivalents of samples were calculated using an extinction coefficient of 1.57×10^5 $M^{-1}cm^{-1}$. The MDA level in the UV-A radiated plant samples were compared with the non UV-A radiated controls.

Experimental design and statistical analysis: Experiment was designed by completely randomized design (CRD) with replicates (n ≥ 3). Statistical analysis for each experiment was performed with one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT). Significant differences among means were determined by LSD test at a P value < 0.05.

Results and Discussion

***PAP1* transgenic enhanced metabolite accumulation in response to lights:** Five lines of *PAP1* transgenic and WT tobaccos were grown under sterile conditions with PAR or PAR+UV-A. Leaf extract was detected for the specific metabolites. The extracts were determined the TSS content and the result is shown in Fig. 2. The additional UV-A radiation enhances the accumulation of TSS to 1.3 times in WT. The average content of TSS in the extracts of transgenic tobacco was approximately 1.5 times of that in WT tobacco under both conditions. However, these extracts showed variables in TSS content depending on the transgenic line. *PAP1* transgenic line P5 grown under PAR and PAR+UV-A conditions showed the highest TSS content (1.72 and 1.67 times of WT, respectively). UV-A enhanced levels of TSS in WT and over-expression of *PAP1*. The increased TSS levels in plants are often associated with environmental stress adaptations in order to mitigate the severity (Rosa *et al.*, 2009).

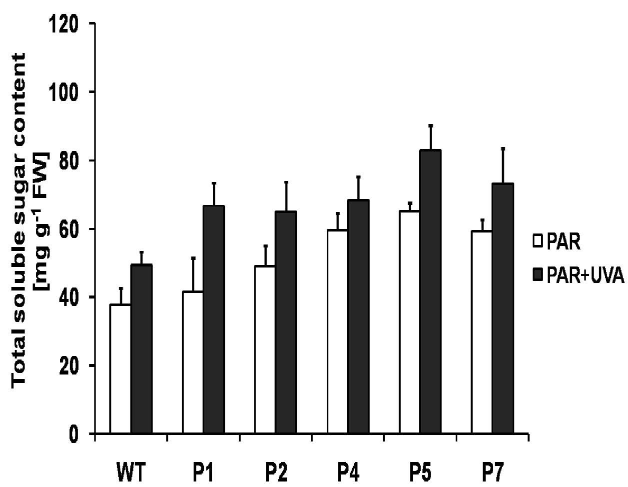

Fig. 2. The total soluble sugar (TSS) content (mg g^{-1} fresh weight) found in the leaf extracts of *PAP1* transgenic and WT plant under PAR or PAR combined with UV-A lights. The error bar indicates the standard deviation from four replicates.

Under PAR, extracts of *PAP1* transgenic contained amounts of almost all detected substances higher than WT tobacco, but the level of these substances are different. Results are shown in Table 1. The levels of substances, especially in the early biosynthesis of flavonoids (naringenin and apigenin) and *p*-coumaric acid, in transgenic were significantly higher (1.6-1.8 times by average) than those of WT. While the levels of kaempferol and pelargonidin in the transgenic extract were slightly different to those in WT extract.

Table 1. Relative amounts of metabolites from phenylpropanoid biosynthesis in leaf extracts of *PAP1* transgenic 5 lines and WT under PAR and PAR+UV-A conditions.

Conditions	Plant lines	Phenylpropanoid derivatives (Abs g^{-1} FW)				
		p-coumaric acid	Naringenin	Apigenin	Kaempferol	Pelargonidin
PAR	WT	4.65 ± 0.8^{a}	4.84 ± 0.7^{a}	4.10 ± 0.8^{a}	0.70 ± 0.05^{a}	0.027 ± 0.003^{a}
	P1	7.66 ± 2.3^{b}	8.21 ± 2.8^{b}	7.31 ± 2.5^{b}	1.06 ± 0.37^{a}	0.044 ± 0.018^{bcd}
	P2	7.04 ± 1.5^{b}	7.61 ± 1.6^{b}	6.81 ± 1.5^{b}	0.88 ± 0.05^{ab}	0.035 ± 0.001^{abc}
	P4	7.78 ± 1.0^{b}	8.57 ± 1.0^{b}	7.61 ± 0.9^{b}	0.83 ± 0.02^{ab}	0.028 ± 0.004^{a}
	P5	7.83 ± 0.3^{b}	8.51 ± 0.2^{b}	7.54 ± 0.2^{b}	1.01 ± 0.13^{abc}	0.042 ± 0.012^{bcd}
	P7	7.87 ± 1.1^{b}	8.85 ± 1.4^{b}	7.93 ± 1.2^{b}	0.85 ± 0.13^{ab}	0.027 ± 0.003^{a}
	PAP1 average	7.64 ± 0.3^{b}	8.35 ± 0.5^{b}	7.44 ± 0.4^{b}	0.93 ± 0.10^{abc}	0.035 ± 0.008^{abc}
PAR+UV-A	WT	6.20 ± 0.4^{a}	6.83 ± 0.7^{ab}	6.01 ± 0.7^{ab}	1.18 ± 0.35^{bcd}	0.033 ± 0.003^{ab}
	P1	11.47 ± 1.0^{cd}	12.76 ± 1.1^{c}	11.61 ± 1.3^{cd}	1.44 ± 0.27^{cde}	0.044 ± 0.002^{bcd}
	P2	11.87 ± 1.2^{cd}	12.88 ± 1.5^{c}	11.58 ± 1.4^{cd}	1.80 ± 0.40^{e}	0.053 ± 0.003^{de}
	P4	10.69 ± 0.2^{c}	11.57 ± 0.3^{c}	10.43 ± 0.3^{c}	1.71 ± 0.35^{e}	0.055 ± 0.005^{de}
	P5	13.13 ± 1.4^{d}	13.93 ± 1.3^{c}	13.04 ± 1.7^{d}	1.76 ± 0.15^{e}	0.058 ± 0.006^{e}
	P7	11.98 ± 0.2^{cd}	13.47 ± 0.4^{c}	12.14 ± 0.4^{cd}	1.49 ± 0.04^{de}	0.047 ± 0.003^{cde}
	PAP1 average	11.83 ± 0.9^{cd}	12.92 ± 0.9^{c}	11.76 ± 0.9^{cd}	1.64 ± 0.2^{e}	0.051 ± 0.006^{de}

The means ± standard deviation (SD) of the results obtained from three biological replicates are shown. Differences in $^{a,b,c\ldots}$ indicated significant differences at $p<0.05$ compared within the same column only

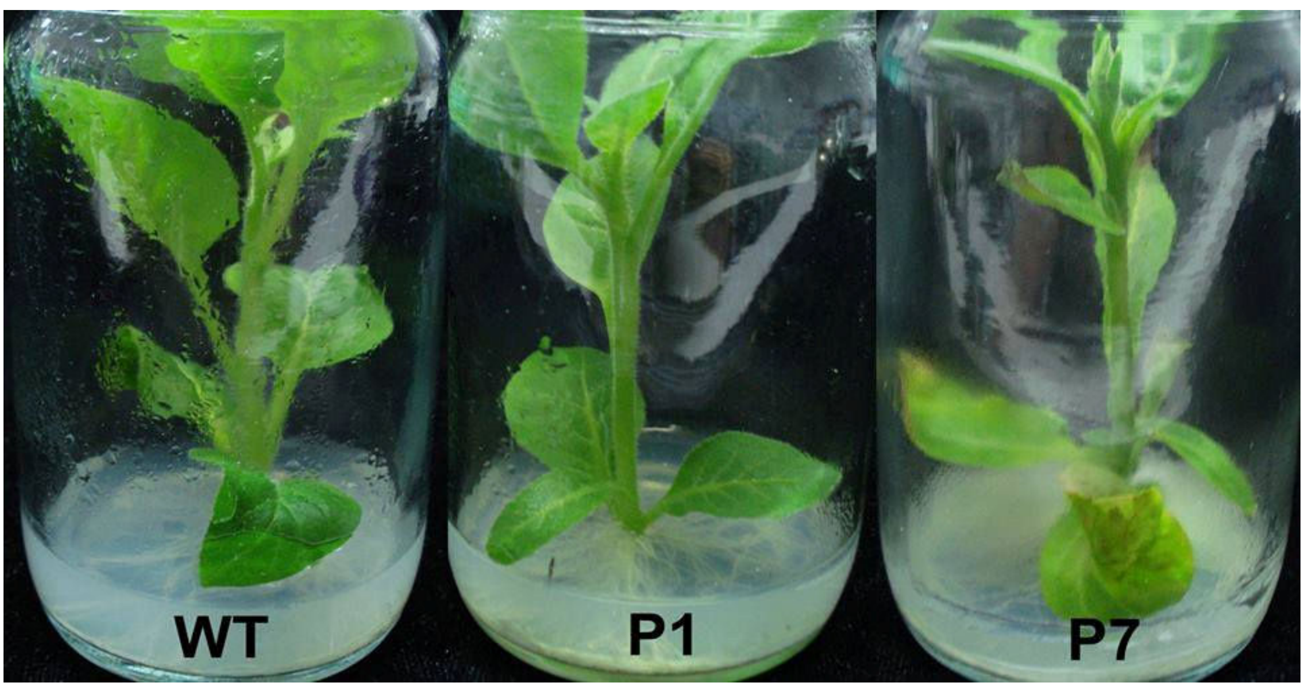

Fig. 3. Phenotypes of three-week-old tobaccos under PAR condition, wild type (WT) and *AtPAP1* transgenic line P1 and P7.

Under 7 days radiation of PAR+UV-A, transgenic and WT tobaccos had not evidently affected phenotypic changes in comparison to themselves grown under PAR alone. In exception, transgenic line P7 showed that it has a high potential to mature and produce flowers under aseptic conditions (Fig. 3.). WT tobacco treated PAR+UV-A showed the significant increases of kaempferol to 1.7 times from WT treated PAR alone, while the levels of *p*-coumaric acid and other flavonoids had no significant increase. The additional UV-A affected different induction of metabolic changes in each transgenic line. In WT and *PAP1* transgenic tobaccos, the relative levels of *p*-coumaric acid, naringenin and apigenin were induced higher than that of flavonol and anthocyanin. However, kaempferol in transgenics was also the highest increase (1.8 times by average) compared to themselves treated under PAR.

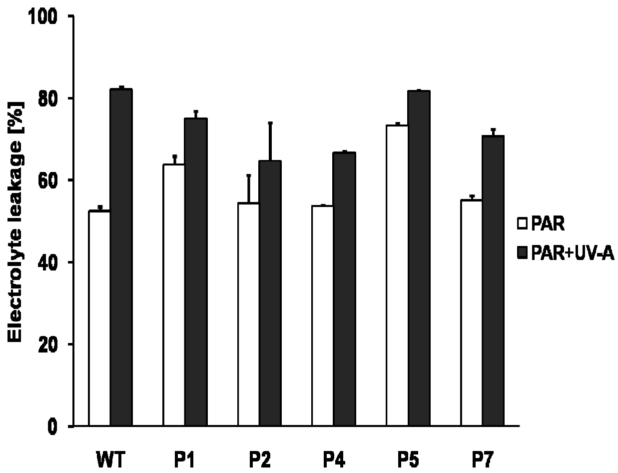

Fig. 4. The electrolyte leakage (%) of leave tissues in *AtPAP1* transgenic lines and WT plant under PAR or PAR combined with UV-A conditions. The error bars indicated the standard deviation (SD) of each line three replicates.

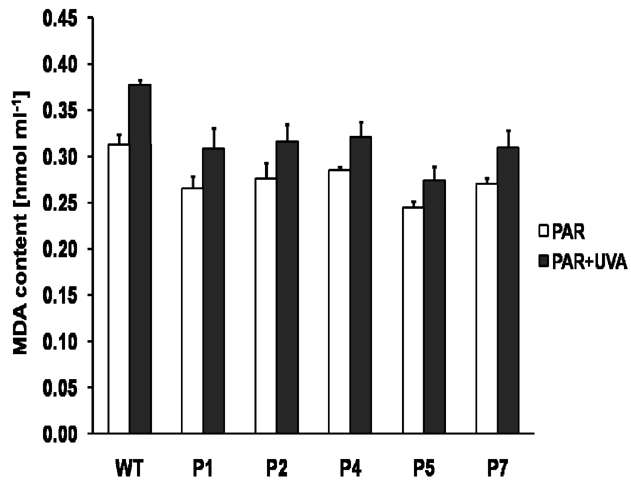

Fig. 5. The malondialdehyde content (nmol ml^{-1}) in *AtPAP1* transgenic and WT leaves under PAR or PAR combined with UV-A conditions. The error bars indicated the standard deviation (SD) of each line five replicates.

***PAP1* transgenic increased cell protection:** The electrical conductivity of electrolytes leaked out from the tissues of WT and *PAP1* transgenic treated with PAR or PAR+UV-A is presented in Fig. 4. The additional UV-A radiation strongly affected electrolytes leaked in WT plant (1.6 times), while fewer changes were found in transgenic (1.1-1.2 times). Under the same UV-A conditions, transgenic line P5 accumulated the highest contents of TSS and flavonoids, and showed the least increase of electrolytes leaked. The electrolytes leaked percentage of this line under the normal PAR condition was the highest. Flavonoids are plant polyphenols which have a high potential in donating electrons to environments including free radicals. Thus transgenic plants containing high flavonoid backgrounds, might give a higher level of electrolyte leakage than normal plants. The highly enhance of electrolyte leakage is considered as a high potential of membrane damage. Our result shows that *PAP1* transgenic treated UV-A slightly increase electrolyte leakage, therefore metabolic production in transgenic should have the protected function to cell membranes. Peroxidation reaction of unsaturated fatty acid yield MDA as one of the final product. This product has been considerate as a maker for stress sensitivity. MDA content was stimulated from leaf tissue extractions in both conditions. Results are presented in Fig. 5. Additional UV-A radiation significantly induced lipid peroxidation, in both WT and transgenics. However, lipid peroxidations in all transgenic were less than that in WT in both conditions and also showed less enhancement under additional UV-A. Transgenic line P5 has the least MDA content under both conditions. This result suggests that *PAP1* transgenic is efficient in reducing MDA products in lipid peroxidation.

The relation between metabolites and plant cell protection: The relationships between substance increase and cell protecting potentials (electrolyte leakage and MDA levels) were analyzed in plants radiated under additional UV-A. UV-A induced the enhancing accumulation of TSS which is slightly correlated with diminishing electrolytes leaked out of tissues (R^2 =0. 16) while it is highly correlated with the rising MDA level (R^2 =0.49). The increased accumulation of apigenin was highly related to the reducing electrolyte leakage (R^2 =0.52) while those of *p*-coumaric acid and naringinin were weakly related (R^2 =0.36 and 0.33, respectively). However, these substances were not directly related to reducing MDA levels in UV-A treated plants. In contrast, increasing kaempferol and pelargonidin are effectively related to reducing level of MDA (R^2 =0.34 and 0.30, respectively). Plant adaptation to additional UV-A is converting primary metabolites into the specific protective metabolites. Our results show that *PAP1* transgenic containing primary (TSS) and secondary metabolites (*p*-coumaric acid and flavonoids) under both condition higher than WT. These transgenics also have less increases of the cell damage factors (ion leakage and lipid peroxidation). It is implied that UV-A induced stress in transgenics is less than that in WT.

The primary metabolites and upstream precursors play a major role in metabolite flux into desirable pathways and provide plant growth and survival. Flavonoid substances excepting anthocyanins absorb different wavelengths in the energetic solar wavelengths (UV region), in consequence; they allow the optimal light energy. Therefore plants accumulated high flavonoid level were less oxidative damage. This result is in concordance with Arabidopsis plants mutated in the flavonoid biosynthetic gene. These plants were found to be excessively sensitive to UV-B. (Li *et al*., 1993; von Wettberg *et al*., 2010).

The UV-A light significantly affected the increasing accumulations of TSS and kaempferol in both WT and *PAP1* transgenics. Kaempferol, a representative of flavonol group, is the most increased substances in response to additional UV-A radiation. This result agrees with the previous experiments that plant flavonol synthesis is induced by environmental factors such as light intensity, light wavelenght and sucrose (Lillo *et al*., 2008; Hofmann *et al*., 2000; Ryan *et al*., 1998). Plants enhance the accumulation of flavonols to absorb UV-A radiation with good quantum efficiency (Goulas *et al*., 2004). Flavonol accumulation may diminish harm from radiation while transmitting PAR to the plant photoreceptors. We postulated that the inducing flavonol biosynthesis is enhanced by additional UV-A, however expression of the *PAP1* gene may slightly increase the response sensitivities. Flavonol biosynthesis may be not directly regulated by the expression of the *PAP1* gene (Rowan *et al*., 2009).

These *PAP1* transgenics increased their response to light by enhancing accumulation of *p*-coumaric acid and flavonoid metabolites but did not result in a strong red/purple plant similar to previous reports (Borevitz *et al*., 2000). However, our result agrees with recent results. They reported that after introduced *PAP1* into canola, tobacco and tomatoes, few transgenic lines enhanced accumulation of these substances, but the larger part of the transgenic lines had the visible phenotype similar to WT (Zhou *et al*., 2008; Xie *et al*., 2006; Li *et al*., 2010). The *PAP1* gene was randomly inserted into the genome position of each transgenic line. Their inserted position may strongly affect to light inducers and also effect the flavonoid accumulations within cells. This result is in agreement with observations of tobacco ectopic expressing *PAP1* and *TT8* (Zhou *et al*., 2008) and also has a similar trend to the reports that species and cultivars of plants slightly divergent in genetics may differ widely in their response to UV-B (Cartwright *et al*., 2001; Murali *et al*., 1988). Nevertheless, the light mechanisms regulating signal transduction and metabolite accumulations are not clear.

The promoters of phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) genes, encoded rate limiting step enzymes in phenylpropanoid and flavonoid pathways, respectively, have been previously identified for the UV-light responsive element (Logemann *et al*., 2000). This *CHS* gene promoter also contains sucrose boxes which were found in the sucrose inducible promoter (Tsukaya *et al*., 1991). Together with the previous reports, this suggests that sugar and light signal pathways closely interact (Smeekens, 2000). *PAP1* encodes a MYB transcription factor which works in a combinatorial complex with bHLH and WD-repeat transcription factors and involves the regulating expression of genes in these pathways (Gonzalez *et al*., 2008; Zimmermann *et al*., 2004). These transcription factors may involve sensing signals and activating biosynthetic pathways (Sompornpailin *et al*., 2002). Sugar stimulating biosynthesis of anthocyanin in different plant organs has been report previously (Hara *et al*., 2003; Weiss, 2000). In concord to the transcript profiling of *Arabidopsis* genome reveals that the pathways of phenylpropanoid and flavonoid are vigorously up-regulated following sucrose treatment (Solfanelli *et al*., 2006). Furthermore, *PAP1* and *TTG1* genes were also essential for the sucrose-induced anthocyanin accumulation in *Arabidopsis* (Shirley *et al*., 1995; Teng *et al*., 2005).

Our experiment first reported that tobacco leaves expressing *PAP1* enhanced TSS. The increasing amount of TSS had a weak relationship with reducing electrolytes leaked out of tissues, but had a strong relationship with increasing MDA levels. Sugars are known as fundamental metabolite producing in response to abiotic stresses. *PAP1* transgenic enhances TSS which may supply precursors and energies into the pathway of protective metabolites such as flavonoids. Furthermore sugars can act as signaling messengers involved in up-regulation and down-regulation of stress-related genes that will benefit the plant in controlling photosynthesis and the ROS balance (Rosa *et al*., 2009; Smeekens, 2000). Soluble sugars seem to have a relating effect on processes both ROSs producing and scavenging systems (Couee *et al*., 2006). On the other hand, in nature flavonoid derivatives are usually found in a glycosylated form (sugar-bound) (Hofmann *et al*., 2000; Wilson *et al*., 1998). Thus, these glycosylated flavonoids may involve in the increase of soluble sugar in plant cells.

In this experiment, the highest increases of flavonol induced by additional UV-A was presented relative with the reducing levels of MDA. The flavonol derivatives increased in leaf epidermal should provide effective UV filters for the mesophyll layer (Fischbach *et al*., 1999; Treutter, 2006). Furthermore, flavonol substances are considered to have strong antioxidant activity due to the scavenging ability of free radicals, therefore it reduced the MDA product of lipid peroxidation (Sakanashi *et al*., 2008; Fahlman & Krol, 2009). Also in agreement with the experiment in human cell, the same group of flavonol substance, quercetin and its glycosylated derivative, is the most efficient in protecting cells from UV radiation by reducing reaction of lipid peroxidation (Pastore *et al*., 2009; Filipe *et al*., 2005; Desentis-Mendoza *et al*., 2006). Furthermore, the other flavonoids have differences in ROSs scavenging activities depending on the functional group of their structure (Agati *et al*., 2012; Prochazkova *et al*., 2011). *p*-coumaric acid, a hydroxycinnamic acid derivatives, has been presented the radical scavenging activities and the membrane preventing from lipid peroxidation (Rice-Evans *et al*., 1996; Grace & Logan, 2000) consequently reduce electrolyte leakage.

Conclusion

Individual line of *PAP1* transgenic significantly enhanced primary metabolites (TSS), and secondary metabolites (*p*-coumaric acid, naringenin and apigenin) under PAR. Additional UV-A radiation affected the increasing accumulation of soluble sugar and flavonol (kaempferol) in WT but significantly affected the increasing accumulation of all detected substances in *PAP1* transgenic. The increasing accumulations of substance at the early pathway (apigenin, naringenin) were highly related to the reducing electrolyte leakage. However the enhancing kaempferol and pelargonidin accumulations effectively related the reducing level of MDA. Thus the tobacco genetic background expressing *PAP1* showed the enhancing light responses via primary metabolites such as sugars and producing the protective secondary metabolites, consequently increasing cell protections.

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References

Agati, G., E. Azzarello, S. Pollastri and M. Tattini. 2012. Flavonoids as antioxidants in plants: Location and functional significance. *Plant Sci.*, 196: 67-76.

Bajji, M., J.M. Kinet and S. Lutts. 2002. The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. *Plant Growth Regul.*, 36: 61-70.

Barthelmebs, L., C. Divies and J.F. Cavin. 2000. Knockout of the *p*-coumarate decarboxylase gene from Lactobacillus plantarum reveals the existence of two other inducible enzymatic activities involved in phenolic acid metabolism. *Appl. Environ. Microbiol.*, 66: 3368-3375.

Blokhina, O., E. Virolainen and K.V. Fagerstedt. 2003. Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Ann. Bot.,* 91: 179-194.

Borevitz, J.O., Y. Xia, J. Blount, R.A. Dixon and C. Lamb. 2000. Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. *Plant Cell.*, 12: 2383-2394.

Bruns, B., K. Hahlbrock and E. Schafer. 1986. Fluence dependence of the ultraviolet-light-induced accumulation of chalcone synthase mRNA and effects of blue and far-red light in cultured parsley cells. *Planta*, 169: 393-398.

Burchard, P. W. Bilger and G. Weissenböck. 2000. Contribution of hydroxycinnamates and flavonoids to epidermal shielding of UV-A and UV-B radiation in developing rye primary leaves as assessed by ultraviolet-induced chlorophyll fluorescence measurements. *Plant Cell Environ.*, 23: 1373-1380.

Cartwright, H.N., C. Baucom, P. Singh, K.L. Smith and A.E. Stapleton. 2001. Intraspecific comparisons reveal differences in the pattern of ultraviolet radiation responses in four maize (*Zea mays* L.) varieties. *J Photochem Photobiol B.*, 62: 88-96.

Chalker-Scott, L. 1990. Environmental significance of anthocyanins in plant stress responses. *Photochem Photobiol.*, 70: 1-9.

Christie, J.M. and G. Jenkins. 1996. Distinct UV-B and UV-A/blue light signal transduction pathways induce chalcone synthase gene expression in *Arabidopsis* cells. *Plant Cell.*, 8:1555-1567.

Cominelli, E., G. Gusmaroli, D. Allegra, M. Galbiati, H.K. Wade, G.I. Jenkins and C. Tonelli. 2008. Expression analysis of anthocyanin regulatory genes in response to different light qualities in *Arabidopsis thaliana*. *J. Plant Physiol.*, 165: 886-894.

Couee, I., C. Sulmon, G. Gouesbet and A. El Amrani. 2006. Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. *J. Exp. Bot.*, 57: 449-459.

Desentis-Mendoza, R.M., H. Hernandez-Sanchez, A. Moreno, R.D.C. Emilio, L. Chel-Guerrero, J. Tamariz and M.E. Jaramillo-Flores. 2006. Enzymatic polymerization of phenolic compounds using laccase and tyrosinase from Ustilago maydis. *Biomacromolecules.*, 7: 1845-1854.

DuBois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, 28: 350-356.

Fahlman, B.M. and E.S. Krol. 2009. Inhibition of UVA and UVB radiation-induced lipid oxidation by quercetin. *J Agric. Food Chem.*, 57: 5301-5305.

Filipe, P., J.N. Silva, J. Haigle, J.P. Freitas, A. Fernandes, R. Santus and P. Morliere. 2005. Contrasting action of flavonoids on phototoxic effects induced in human skin fibroblasts by UVA alone or UVA plus cyamemazine, a phototoxic neuroleptic. *Photochem Photobiol Sci.*, 4: 420-428.

Fischbach, R.J., B. Kossmann, H. Panten, R. Steinbrecher, W. Heller, H.K. Seidlitz, H. Sandermann, N. Hertkorn and J.P. Schnitzler. 1999. Seasonal accumulation of ultraviolet-B screening pigments in needles of Norway spruce (*Picea abies* (L.) Karst.). *Plant Cell Environ.*, 22: 27-37.

Gonzalez, A., M. Zhao, J.M. Leavitt and A.M. Lloyd. 2008. Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in *Arabidopsis* seedlings. *Plant J.*, 53: 814-827.

Goulas, Y., Z.G. Cerovic, A. Cartelat and I. Moya. 2004. Dualex: a new instrument for field measurements of epidermal ultraviolet absorbance by chlorophyll fluorescence. *Appl. Opt.*, 43: 4488-4496.

Grace, S.C. and B.A. Logan. 2000. Energy dissipation and radical scavenging by the plant phenylpropanoid pathway. *Philos Trans R Soc Lond B Biol Sci.*, 355: 1499-1510.

Hara, M., K. Oki, K. Hoshino and T. Kuboi. 2003. Enhancement of anthocyanin biosynthesis by sugar in radish (*Raphanus sativus*) hypocotyl. *Plant Science*, 164: 259-265.

Harborne, J.B. 1998. Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis Chapman and Hall, London, UK.

Hodges, D.M., J.M. DeLong, C.F. Forney and R.K. Prange. 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta.*, 207: 604-611.

Hofmann, R.W., E.E. Swinny, S.J. Bloor, K.R. Markham, K.G. Ryan, B.D. Campbell, B.R. Jordan and D.W Fountain. 2000. Responses of nine Trifolium repens L. populations to ultraviolet-B radiation: Differential flavonol glycoside accumulation and biomass production. *Ann. Bot.*, 86: 527-537.

Jin, H. and C. Martin. 1999. Multifunctionality and diversity within the plant MYB-gene family. *Plant Mol Biol.*, 41: 577-585.

Kanthang, S. and K. Sompornpailin. 2013. Increasing Plant Flavonoid Biomaterials in Response to UV-A Light. *Advanced Materials Research.*, 802: 74-78.

Landry, L.G., C.C. Chapple and R.L. Last. 1995. *Arabidopsis* mutants lacking phenolic sunscreens exhibit enhanced ultraviolet-B injury and oxidative damage. *Plant Physiol.*, 109: 1159-1166.

Li, J., T.M. Ou-Lee, R. Raba, R.G. Amundson and R.L. Last. 1993. *Arabidopsis* flavonoid mutants are hypersensitive to UV-B irradiation. *Plant Cell.*, 5: 171-179.

Li, X., M.J. Gao, H.Y. Pan, D.J. Cui and M.Y. Gruber. 2010. Purple canola: *Arabidopsis* PAP1 increases antioxidants and phenolics in *Brassica napus* leaves. *J. Agric. Food Chem.*, 58: 1639-1645.

Lillo, C., U.S. Lea and P. Ruoff. 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.

Logemann, E., A. Tavernaro, W. Schulz, I.E. Somssich and K. Hahlbrock. 2000. UV light selectively coinduces supply pathways from primary metabolism and flavonoid secondary product formation in parsley. *Proc. Natl. Acad. Sci. U S A.*, 97: 1903-1907.

Maness, N. (ed). 2010. Extraction and analysis of soluble carbohydrate vol 693. Plant Stress Tolerance, Methods in Molecular Biology E-Publishing Inc, New York.

Murali, N.S., A.H. Teramura and S.K. Randall. 1988. Response differences between two soybean cultivars with contrasting UV-B radiation sensitivities. *Photochem Photobiol*., 48: 653-657.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol. Plant*., 15: 473-497.

Pastore, S., A. Potapovich, V. Kostyuk, V. Mariani, D. Lulli, C. De Luca and L. Korkina. 2009. Plant polyphenols effectively protect HaCaT cells from ultraviolet C-triggered necrosis and suppress inflammatory chemokine expression. *Ann. N.Y. Acad. Sci*., 1171: 305-313

Prochazkova, D., I. Bousova and N. Wilhelmova. 2011. Antioxidant and prooxidant properties of flavonoids. *Fitoterapia*., 82: 513-523.

Rice-Evans, C.A., N.J. Miller and G. Paganga. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med*., 20: 933-956.

Robberecht, R. and M.M. Caldwell. 1978. Leaf epidermal transmittance of ultraviolet radiation and its implications for plant sensitivity to ultraviolet-radiation induced injury. *Oecologia*., 32: 277-278.

Rosa, M., C. Prado, G. Podazza, R. Interdonato, J.A. Gonzalez, M. Hilal and F.E. Prado. 2009. Soluble sugars--metabolism, sensing and abiotic stress: a complex network in the life of plants. *Plant Signal Behav*., 4: 388-393.

Rowan, D.D., M. Cao, K. Lin-Wang, J.M. Cooney, D.J. Jensen, P.T. Austin, M.B. Hunt, C. Norling, R.P. Hellens, R.J. Schaffer and A.C. Allan. 2009. Environmental regulation of leaf colour in red 35S:PAP1 *Arabidopsis thaliana*. *New Phytol*., 182: 102-115.

Ryan, K.G. K.R. Markham, S.J. Bloor, J.M. Bradley, K.A. Mitchell and B.R. Jordan. 1998. UVB radiation-induced increase in quercetin:kaempferol ratio in wild-type and transgenic lines of Petunia. *Photochem. Photobiol*., 68: 323-330.

Sakanashi, Y., K. Oyama, H. Matsui, T.B. Oyama, T.M. Oyama, Y. Nishimura, H. Sakai and Y. Oyama. 2008. Possible use of quercetin, an antioxidant, for protection of cells suffering from overload of intracellular Ca^{2+}: A model experiment. *Life Sciences*., 83: 164-169.

Shirley, B.W., W.L. Kubasek, G. Storz, E. Bruggemann, M. Koornneef, F.M. Ausubel and H.M. Goodman. 1995. Analysis of *Arabidopsis* mutants deficient in flavonoid biosynthesis. *Plant J*., 8: 659-671.

Smeekens, S. 2000. Sugar-Induced Signal Transduction in Plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol*., 51: 49-81.

Solfanelli, C., A. Poggi, E. Loreti, A. Alpi and P. Perata. 2006. Sucrose-specific induction of the anthocyanin biosynthetic pathway in *Arabidopsis*. *Plant Physiol*., 140: 637-646.

Sompornpailin, K., Y. Makita, M. Yamazaki and K. Saito. 2002. A WD-repeat-containing putative regulatory protein in anthocyanin biosynthesis in *Perilla frutescens*. *Plant Mol. Biol*., 50: 485-495.

Stapleton, A.E. and V. Walbot. 1994. Flavonoids can protect maize DNA from the induction of ultraviolet radiation damage. *Plant Physiol*., 105: 881-889.

Teng, S., J. Keurentjes, L. Bentsink, M. Koornneef and S. Smeekens. 2005. Sucrose-specific induction of anthocyanin biosynthesis in *Arabidopsis* requires the MYB75/PAP1 gene. *Plant Physiol*., 139: 1840-1852.

Tohge, T., K. Matsui, M. Ohme-Takagi, M. Yamazaki and K. Saito. 2005. Enhanced radical scavenging activity of genetically modified *Arabidopsis* seeds. *Biotechnol. Lett*., 27: 297-303.

Treutter, D. 2006. Significance of flavonoids in plant resistance: a review. *Environmental Chemistry Letters*., 4: 147-157.

Tsukaya, H., T. Ohshima, S. Naito, M. Chino and Y. Komeda. 1991. Sugar-Dependent Expression of the CHS-A Gene for Chalcone Synthase from Petunia in Transgenic *Arabidopsis*. *Plant Physiol*., 97: 1414-1421.

Von Wettberg, E.J., M.L. Stanton and J.B. Whittall. 2010. How anthocyanin mutants respond to stress: the need to distinguish between stress tolerance and maximal vigour. *Evolutionary Ecology Research*., 12: 457-476.

Wade, H.K., T.N. Bibikova, W.J. Valentine and G.J. Jenkins. 2001. Interactions within a network of phytochrome, cryptochrome and UV-B phototransduction pathways regulate chalcone synthase gene expression in *Arabidopsis* leaf tissue. *Plant J*., 25: 675-685.

Weiss, D. 2000. Regulation of flower pigmentation and growth: multiple signaling pathways control anthocyanin synthesis in expanding petals. *Physiologia Plantarum*., 110: 152-157.

Wilson, K.E., M.I. Wilson and B.I. Greenberg. 1998. Identification of the flavonoid glycosides that accumulate in *Brassica napus* L. cv. Topas specifically in response to ultraviolet B radiation. *Photochem Photobiol*., 67: 547-553.

Xie, D.Y., S.B. Sharma, E. Wright, Z.Y. Wang and R.A. Dixon. 2006. Metabolic engineering of proanthocyanidins through co-expression of anthocyanidin reductase and the PAP1 MYB transcription factor. *Plant J*., 45: 895-907.

Zhang, F., A. Gonzalez, M. Zhao, C.T. Payne and A. Lloyd. 2003. A network of redundant bHLH proteins functions in all TTG1-dependent pathways of *Arabidopsis*. *Development*., 130: 4859-4869.

Zhou, L.L., H.N. Zeng, M.Z. Shi and D.Y. Xie. 2008. Development of tobacco callus cultures over expressing *Arabidopsis* PAP1/MYB75 transcription factor and characterization of anthocyanin biosynthesis. *Planta*., 229: 37-51.

Zimmermann, I.M., M.A. Heim, B. Weisshaar and J.F. Uhrig. 2004. Comprehensive identification of *Arabidopsis thaliana* MYB transcription factors interacting with R/B-like bHLH proteins. *Plant J*., 40: 22-34.

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