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TOBACCO RANDOMLY INSERTED *TT8* DIFFERENLY ENHANCE LIGHT SIGNALS AND FLAVONOID ACCUMULATION

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Abstract

The individual lines of tobacco over-expressing *TT8*, a bHLH gene, were constructed and cultured under tissue culture condition radiating with photosynthetically activation radiation (PAR) or PAR+UVA. They were compared to wild type (WT). Leaf of treated plants was extracted and analyzed for flavonoid accumulations using a spectrophotometer. The extract of *TT8* plants significantly contained flavone, flavonol and anthocyanin level, higher than the WT extract did. The petal extracts of mature transgenic under PAR had a similar absorbance profile of each substance, but these extracts had higher flavonoid contents than the leaf extracts did. All flavonoid subgroups and *p*-coumaric acid biosynthesis were significantly enhanced after the additional UVA radiation to plant. This UVA condition slightly stimulated an accumulation of these substances in normal plant. Some transgenic greatly increased flavonoid accumulation in responding to PAR+UVA, but the others were slightly different compared to WT. The distinct insertion site is directly affected *TT8* gene expression. Transgenic seeds had a dark brown color more than WT seed, which indicated high content of polymer flavonoids (proanthocyanins). This over-expressing *TT8* in transgenic tobacco may directly or indirectly enhance the signal transductions of PAR and UVA and raise up flavonoid accumulation.

Key words: bHLH; Flavonoids; Lights; *TT8*; UVA.

Introduction

Flavonoids accumulated in epidermal plant cells have a light shading function, thus they mitigate harmful effects to plant by reducing the intensity and energy of the lights (Harborne & Williams, 2000; Zeng *et al*., 2010). Strong sunlight and ultraviolet (UV) radiation induce the generation of free radicals and provoke the expression of genes in flavonoid biosynthesis in plants (Casati & Walbot, 2003; Mol *et al*., 1996; Zimmermann *et al*., 2004). Different flavonoid subgroups have the distinct function in reducing the effect of radical damages in the plant cell (Chutipaijit *et al*., 2012; Gould *et al*., 1995; Neill & Gould, 2003; Steyn *et al*., 2002) and may involve in cascaded transduction of signals (Ferrer *et al*., 2008). Genes and regulatory proteins in the biosynthesis of flavonoid pigments are shown in Fig. 1. Flavonoid biosynthesis are stimulated by internal and external factors via transcription factors (TFs). TFs, such as bHLH, R2R3-MYB and WD-repeat, have been reported to form a complex together and regulate the specific gene promoters in flavonoid biosynthesis (Morita *et al*., 2006; Quattrocchio *et al*., 1999; Walker *et al*., 1999; Winkel-Shirley 2001; Zhang *et al*., 2003). TT8, a bHLH protein family, has been found to regulate the expression of the BANYULS (BAN) and DIHYDROFLAVONOL 4-REDUCTASE (DFR) proteins which are involved in proanthocyanin and anthocyanin metabolisms (Nesi *et al*., 2000; Zhang *et al*., 2003; Zimmermann *et al*., 2004).

Ultraviolet-B (UVB: 280 to 320 nm) is severely harmful to plant physiology while ultraviolet-A (UVA: 320 to 400 nm) has a lesser energy of radiation, but significant in controlling plant developments (Bjorn 1994; Jansen *et al*., 1998; Rozema *et al*., 1997). UVA has been shown to enhance an expression of *chalcone synthase (CHS)* gene in flavonoid biosynthesis more than UVB (Bruns *et al*., 1986; Christie & Jenkins 1996). Plant mutated in genes encoded for CHS or chalcone isomerase (CHI) enzymes were sensitive to UV radiation (Li *et al*., 1993).

Several plants mutated at the *bHLH* gene and also *TT8* positions have been reported to be associated in accumulation of flavonoids but plants over-expressing *bHLH* have been presented an alternative result. Moreover, these plants did not clearly show an enhancing signal the accumulation of flavonoids in leaf (Heim *et al*., 2003; Nesi *et al*., 2000; Zhang *et al*., 2003). Many researchers often selected the transgenic that yielded the most visible characteristics, but might not be suitable representatives for gene functional study. In contrast to the others, our study used all transgenic and broad spectrum of the spectrophotometry technique for evaluating flavonoid substances. Our findings showed that transgenic tobacco over-expressing TT8 increased light response and enhanced flavonoid accumulations in the tissues. Moreover, TT8 transgenic increase the synthesis of different flavonoid subgroups, not reported in the previous studies.

Materials and Methods

Transgenic construction and light treatment: *TT8* fragments (AJ277509, GenBank: http://www.ncbi.nlm.nih.gov) were magnified from the template of *Arabidopsis thaliana* cDNA using F primer, 5'-CGGGATCCATGGATGAATCAAGTATTAT -3' and R primer 5'-CGGGATCCATGATTAGTATCATGTATTA-3'. A fragment of the open reading frame (ORF) was ligated into the cloning vector. The DNA sequencing technique was used for confirming the nucleotide sequence. This *TT8* fragment was sub-cloned into pBI121 vector (accession number AF485783). This plasmid was transformed into *Agrobacterium tumefaciens* by using a freeze throw method. *A. tumefaciens* containing *TT8* plasmid was used for tobacco (*Nicotiana tabacum*) infection by the leaf disk method (Horsch *et al*., 1985). Plantlets regenerated on MS medium (Murashige & Skoog 1962) containing 100 mgl^{-1} kanamycin were collected and amplified in normal condition. Genomic DNA of each collected plant was extracted and used for determining the

TT8 fragment insertion using PCR techniques. The amplified fragments were examined on agarose gel stained with ethidium bromide. This gel was exposed to UV light and recorded an image. The amounts of *TT8* transgenic and wild type (WT) were separately amplified under aseptic condition. Three-week-old plants (WT and *TT8* transgenic) were grown under tissue culture condition at 25±2°C. Two light conditions (photosynthetically activating radiation (PAR, 400 to 700 nm) or the combination of PAR and UVA (PAR+UVA)) were radiated to plants, in a 16 h/8 h light regime at 60 µmol m^{-2} s^{-1} for one week. Leaf was frozen and kept for further substance analysis.

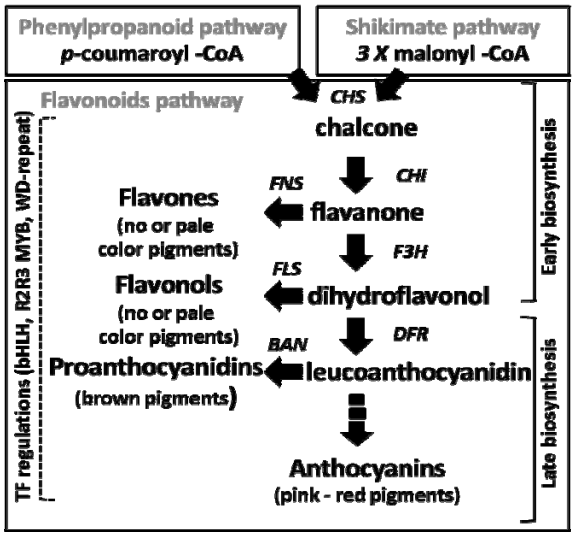

Fig. 1. A biosynthetic scheme of the flavonoid pigments. The italic letter refers to a gene encoded for enzyme as follows: *CHS*, chalcone synthase; *CHI*, chalcone isomerase; *FNS*, flavone synthase; *F3H*, flavanone 3-hydroxylase; *FLS*, flavonol synthase; *DFR*, dihydroflavonol reductase; *BAN*, leucoanthocyanidin reductase. Dash arrow represents a multi-step of enzymes to produce substances.

Accumulation analysis of flavonoid: Powder of frozen sample (0.5 g Fresh weight (FW)) was extracted with 2 ml solvent of water and 1% HCl in methanol (2:3). Leaf extract was removed chlorophylls by extracting with chloroform. Spectrophotometry at specific wavelength was used to analyze the absorbance level of individual flavonoid derivatives and precursor (*p*-coumaric acid) in the extract (Barthelmebs *et al*., 2000, Harborne, 1998). The absorbance value of each sample was calculated and presented in the g FW. The mean with SE of repeated experiments was shown. Data amount treatments were compared using the Independent Samples T-test and Duncan's multiple range test analysis of variance test. Statistical probability ≤ 0.05 was considered significant.

Plant characteristics: Five line transgenics (F_0) containing a high content of different flavonoid subgroups under tissue culture condition radiated with PAR and UVA were selected. Plant derived from aseptic condition was hardened and transferred to soil moistened with Hoagland solution (Hoagland & Arnon, 1950). The culture temperature was the same as tissue culture condition. Plants were irradiated with PAR at 80 µmol m^{-2} s^{-1}. Changes in transgenic phenotypes were examined. Flavonoid subgroups and precursor accumulating in a petal extract of each plant were detected by using the previous analytical method. Flower colors were recorded as a picture. Plants (F_0) were self-fertilized. The mature fertilized seeds (F_1 seeds) were evaluated for color intensity.

Results and Discussion

Plant characteristics under tissue culture condition: The transformed cell regenerated and developed into plantlet on kanamycin containing medium. Each clone of transgenic plant was propagated in tissue culture condition and screened for inserting *TT8* fragment into genome using PCR techniques. The *TT8* amplified results of nineteen independent transgenic are shown in Fig. 2. The visible characteristics of transgenic under tissue condition are similar to those of WT. However, some line of *TT8* transgenic produced dark green leaf and grew at a slower rate that other.

Wild type and transgenic containing *TT8* were grown under PAR or PAR+UVA in tissue culture system for 1 week. Leaf of treated plant was extracted and analyzed for the representative subgroups of flavonoid and *p*-coumaric acid. The substance level in the leaf extract of WT and each *TT8* transgenic lines is presented in Fig. 3. The average amount of substances in transgenic extract compared to WT is presented in Table 1. The extracts of *TT8* transgenic grown under PAR had the average amounts of intermediate substances (flavanone) and *p*-coumaric acid similar to those of WT. However, amounts of flavone, flavonol and anthocyanin in *TT8* extracts were significantly higher (1.1 folds) than those in WT extract. Our experimental result confirmed that *TT8* transgenic affected an increase accumulation of these 3 substances in the leaf. Until now, the evidence at the level of flavone, flavonol substances elevated in this part has rarely been given. Since slightly increased amount of these substances in the leaf was hardly distinguished phenotypic evidence.

The extract of WT and *TT8* plants grown under PAR+UVA condition had the average amounts of flavonoid higher than the extract of same plants grown under PAR condition. The extracts of *TT8* transgenic under PAR+UVA condition significantly had the contents of flavonoid and precursor higher than those of WT plants treated with the same condition and also those of *TT8* plants under PAR condition. Individual transgenic line differently increased levels of flavonoid substances responded to additional UVA. Approximately half number of *TT8* plants was highly increased flavonoid accumulation after UVA radiation. These plant extracts yielded 1.5-2.0 folds more *p*-coumaric acid and flavonoid substances than those extracts of plant under PAR alone. Lines of *TT8* plant which have dark green leaves showed a large amount of flavonoid content in the extracts under both conditions.

DNA transformation using *Agrobacterium* techniques can insert genes randomly into different genome position. It may cause a difference in the gene expression. All individual lines of TT8 transgenic were propagated and used as the replicate in the experiment under controllable environment. Therefore, this result of flavonoid accumulation in transgenic was actually affected by *TT8* gene function.

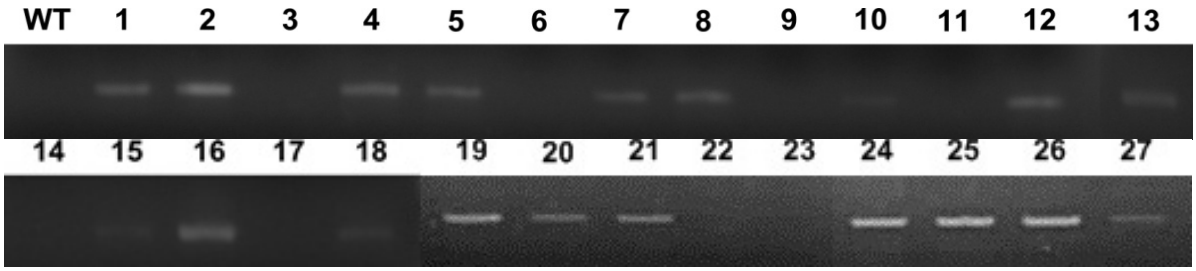

Fig. 2. The PCR amplification result of TT8 fragments using genomic DNA of different transgenic lines (1-27) or wild type (WT) plants as a template.

Our experiment demonstrated that anthocyanin accumulation enhanced in leaf tissue of *TT8* transgenic, while previous reports have not shown that an increasing expression of bHLH proteins alone in transgenic clearly enhanced flavonoid biosynthesis in leaf tissue (Bai *et al*., 2011; Nesi *et al*., 2000; Ramsay *et al*., 2003). Previous general experiments on flavonoid biosynthesis have usually selected transgenic containing visible pigments (anthocyanins and proanthocyanins) and used a few, specific, detection methods for some substance which did not cover all of the biosynthesis. Moreover, plant leaf is generally masked with a plenty of chlorophyll pigments, thus the slight increase of colorless, pale or flavonoid pigments in bHLH transgenic may be difficult to distinguish. Another aspect is that flavonoids in several plants are a large group of substances, and each substance is also produced in different amount which makes it difficult to detect simultaneously by the available analytical methods. The spectrophotometry method may not appropriate to analyze the specific type of flavonoid, but it should be suitable for screening different subgroups and levels of flavonoids in the plant extract. These detecting profiles of several substances in the transgenic extracts could be used as criteria for screening the high potential accumulation of flavonoids.

The amounts of all flavonoid subgroups and precursor in the extracts of plants radiated with PAR+UVA were statistically different with those of the plant radiated with PAR. This result was in agreement with those of different cultivars of soybean radiated with both visible and UV lights had a high accumulation of flavonoids (Winter & Rostas, 2008). Many research works indicate that the expression of several genes involved in phenylpropanoid and flavonoid biosynthesis is induced by UVA radiation (Christie & Jenkins 1996; Wade *et al*., 2001; Zhou *et al*., 2007). However, individual line of transgenic in this experiment had the various responses to UVA light and accumulated diverse qualities and quantities of the substances. Therefore, a randomly genome position inserted gene, significantly affects on the accumulation of flavonoids especially under PAR+UVA condition. Flavonoids synthesized in plant radiated with UV can protect the plants from harmful radiation (Kolb *et al*., 2001). However, the way that light signals regulate gene expression in biosynthesis is still not clear.

Characteristics of mature plant: Five lines of *TT8* transgenic (no. 1, 4, 5, 10 and 24) showed the relative high accumulation of flavonoids in responding to additional UVA. These transgenic lines were selected for analyzing the mature characteristics. The mature transgenics showed normal growth similar to WT but different in color intensity of leaf and petal tissues. Each stage of flowers derived from line no. 4 was used as the representative incomparable to those from WT (Fig. 4a, b). The color intensities of petal flower and filament stamens from *TT8* mature plant were darker than those from WT. This evidence suggested high anthocyanin accumulation in parts of *TT8* flower.

The petal tissues of WT and five *TT8* plants under PAR condition were used for analysis because of high flavonoid accumulation. The absorbance level of each flavonoid subgroup in the extract is shown in Table 2. The extracts of TT8 petal tissues had higher levels of flavone, flavonol and anthocyanin than the extracts of WT petal tissues had. The extract of TT8 petal tissues contained the pigmented anthocyanin nearly 2 folds higher than the extract of WT petal tissues. Interestingly, subgroups of flavonoids increased in the petal transgenic tissues were the same as those in the transgenic leaf under tissue culture condition. However, the petal tissue had higher levels of substance accumulation.

F_1 seeds from transgenic plants were collected and compared to seed from WT. The seed ratio of light brown/dark-brown color produced by WT and transgenic plant are shown in Fig. 4c, f. Approximately 2 times more seeds from the transgenic were dark-brown, compared to those from the WT. High accumulation of proanthocyanins is known to increase the color intensity of the seed coat.

Our experiment showed that proanthocyanins and anthocyanin were significantly enhanced in flower and seed of *TT8* plant. This evidence is similar to other reports of tobaccos expressing bHLH TFs. These transgenic tobaccos promoted the regulation of gene at the late flavonoid biosynthetesis in corolla and seed tissues and resulted an increase of color intensity (Hichri *et al*., 2011; Nesi *et al*., 2000; Ramsay *et al*., 2003). The pigmented flavonoids in petal tissues of flower are easier to identify because of less chlorophyll content. Moreover, this tissue accumulates high flavonoid content under normal condition. The extraordinary substances (flavone and flavonal) found in both leaf and petal of TT8 transgenics were reported in our experiment.

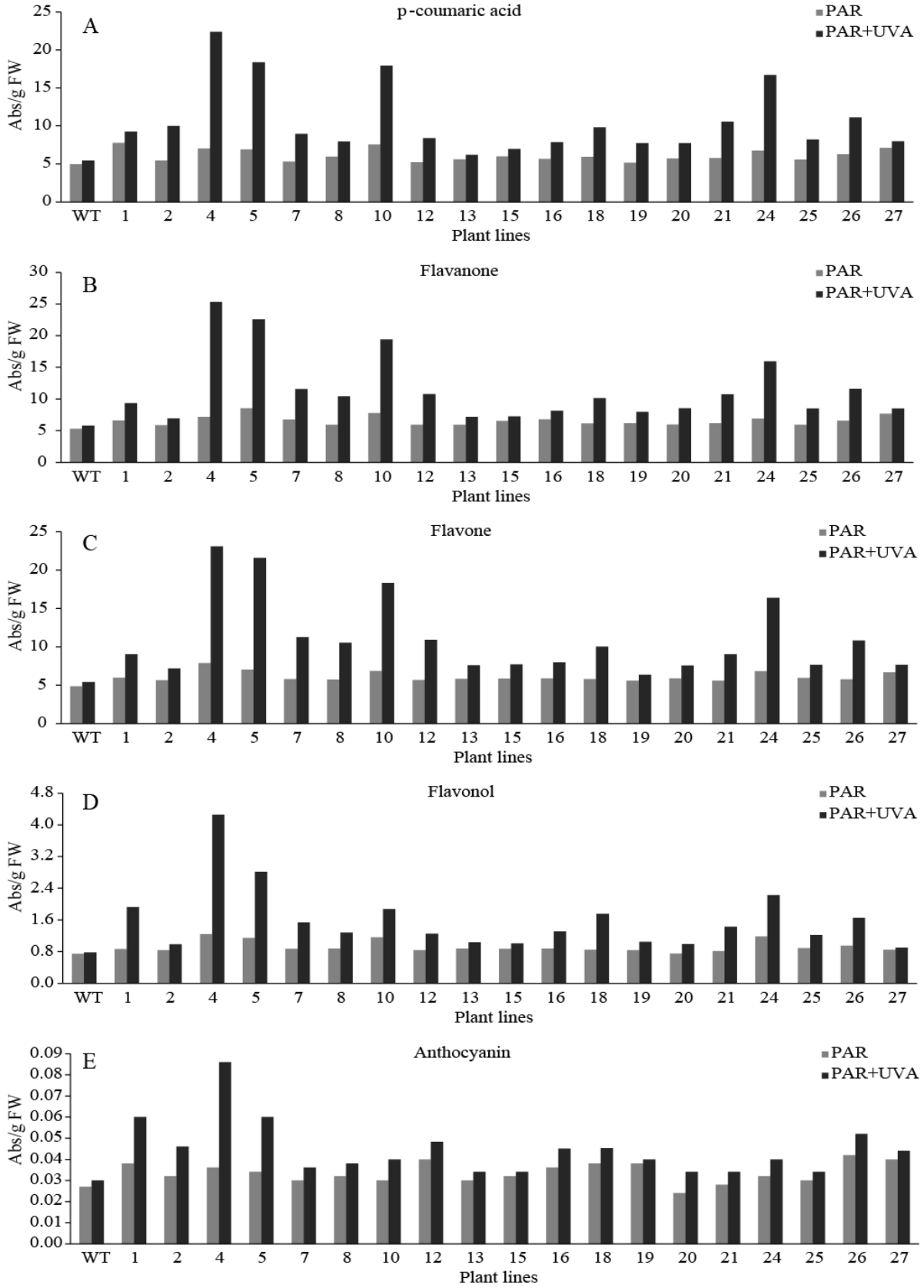

Fig. 3. Flavonoid and *p*-coumaric acid profiles in the extracts of leave tissues from wild type (WT) and each *TT8* transgenic lines grown in tissue culture systems under PAR or PAR+UVA conditions. Data are mean values (n=3).

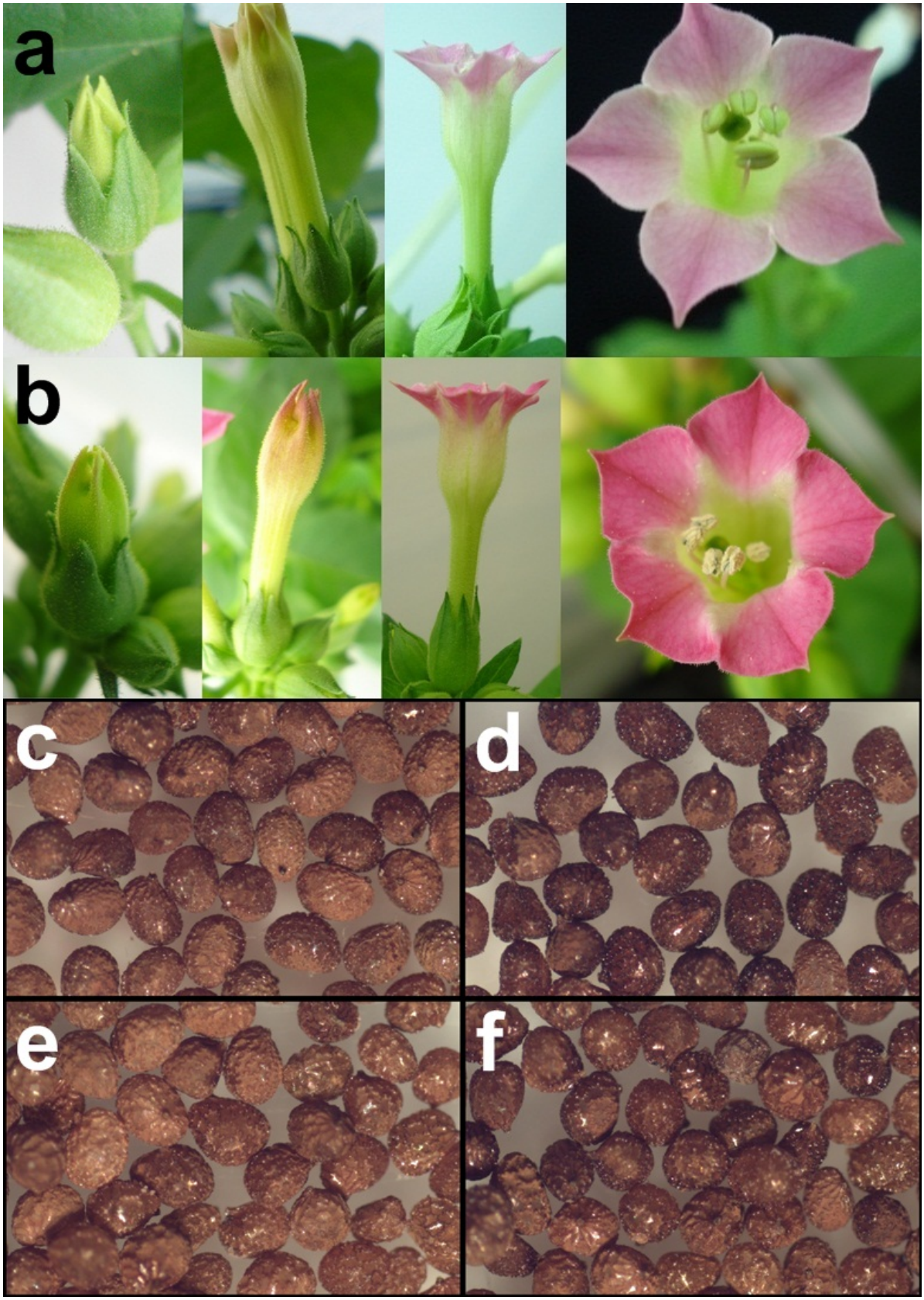

Fig. 4. The flowers at different developmental stages of WT (a) and *TT8* transgenic tobaccos (b). Light brown/dark brown seed ratios produced from WT (c and d 290:70) and *TT8* transgenic (e and f 225:140) tobaccos.

Table 1. Relative contents of flavonoids and *p*-coumaric acid in the extracts of leave tissues from WT and *TT8* transgenic plants grown in tissue culture systems under PAR or PAR+UVA conditions.

Light	Plant	Substance contents				
		***p*-coumaric acid**	**Flavanone**	**Flavone**	**Flavonol**	**Anthocyanin**
PAR	WT	5.78 ± 0.7^{a}	6.31 ± 1.0^{a}	5.09 ± 1.0^{a}	0.81 ± 1.1^{a}	0.029 ± 0.003^{a}
	TT8	6.14 ± 0.8^{a}	6.60 ± 0.7^{a}	6.11 ± 1.3^{b}	0.92 ± 1.2^{b}	0.034 ± 0.005^{b}
PAR+UVA	WT	7.30 ± 0.9^{b}	7.78 ± 1.4^{b}	6.60 ± 1.1^{b}	0.97 ± 1.1^{b}	0.033 ± 0.003^{b}
	TT8	10.74 ± 4.6^{c}	11.62 ± 5.3^{c}	11.08 ± 5.0^{c}	1.60 ± 1.4^{c}	0.045 ± 0.013^{c}

Data (Absorbance/g FW) are mean ± SD values ($n \geq 10$). Differences in letter (a, b, c) indicated significant differences at $p<0.05$ compared within the same derivative

Table 2. Relative contents of flavonoids and *p*-coumaric acid in the extracts of petal tissues from WT and *TT8* transgenic plants grown in the culture room under PAR condition.

Plant	Substance contents				
	***p*-coumaric acid**	**Flavanone**	**Flavone**	**Flavonol**	**Anthocyanin**
WT	77.8 ± 8.2^{a}	78.2 ± 6.4^{a}	70.2 ± 5.7^{a}	29.0 ± 2.3^{a}	1.05 ± 0.2^{a}
TT8	86.8 ± 2.8^{a}	93.9 ± 3.0^{a}	87.5 ± 6.5^{b}	40.1 ± 3.7^{b}	2.90 ± 0.3^{b}

Data are mean ± SD values ($n = 5$). Differences in letter (a, b) indicated significant differences at $p<0.05$ compared within the same derivative

Plant enhanced the expression of *TT8* was not adequate for activating of flavonoid pathway. The other inducers especially light signals is also important. bHLH *Lc* Petunia radiated with strong light were greatly expressed *CHS*, *CHI*, *DFR* and *ANS* genes (Albert *et al*., 2009). UVA radiation greatly induced the difference of flavonoid accumulations in independent line of *TT8* transgenic. However, this radiation slightly effects among lines of *PAP1* transgenic (Sompornpailin & Kanthang, 2015). bHLH proteins are capable to combine with other TFs (Shi & Xie, 2010). TT8 TF interacted with TTG1 (WD-repeat TF) and TT2, PAP1 (R2R3 MYB TFs), this complex has been reported to regulate the flavonoid biosynthetic pathway (Baudry *et al*., 2004; Nesi *et al*., 2000; Nesi *et al*., 2001). bHLH proteins found in various eukaryotic cells are commonly function in signals responding processes. This evidence resulted in signal transduction and effected the regulation of genome transcriptions (Feller *et al*., 2011). WD-repeat protein has the potential to combine with bHLH protein and may modify the bHLH activities (Sompornpailin *et al*., 2002). From our experiment, we postulated that TT8 protein complex may involve in signal transduction of PAR and UVA lights and regulate the expression of flavonoid biosynthetic genes in the plant.

Conclusion

Transgenic over-expressing *TT8* increased the responses of both PAR and PAR+UVA under tissue culture condition. Under PAR+UVA condition, *p*-coumaric acid and flavonoid accumulations were highly induced in *TT8* transgenic leaf, but slightly induced in WT leaf under tissue culture condition. The *TT8* inserted positions in genome importantly affected flavonoid biosynthesis and accumulation. This experiment presented that *TT8* transgenic enhanced light signals and increased the accumulations of flavones and flavonols in leaf and petal tissues in addition to anthocyanins. *TT8* transgenic in normal PAR condition produced more dark-brown seed of high proanthocyanin content. Therefore TT8 proteins are involved in the transductions of PAR and UVA signals that regulates the flavonoid accumulation.

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