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ORIGINAL PAPER

Development of *AtMYB12*-expressing transgenic tobacco callus culture for production of rutin with biopesticidal potential

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Abstract Flavonoids synthesized by the phenylpropanoid pathway participate in a number of physiological and biochemical processes in plants. Flavonols, among flavonoids, are considered as health-protective components in functional foods and they protect plants against certain insect pests. There have been efforts to develop strategies for the enhanced production of flavonols in plants, but limited success was achieved due to complex regulation and poor substrate availability. In the present study, we have developed and optimized method for callus cultures for transgenic tobacco line expressing a flavonol-specific transcription factor, *AtMYB12*, with an objective to use callus as an alternative source of rutin. Transgenic callus displayed enhanced expression of genes related to biosynthetic pathway leading to increased accumulation of flavonols, especially rutin. At each time point of callus growth, the rutin content of transgenic callus was several folds higher than that of wild-type tobacco callus. Supplementation of semi-synthetic diet with extract from transgenic callus as well as purified rutin led to mortality and growth reduction in the *Spodoptera litura* and *Helicoverpa armigera* larvae. This study suggests the biotechnological potential of *AtMYB12*-expressing callus cultures for the production of rutin, which can be used for biopesticide formulations against insect pests.

Key message Tobacco callus cultures expressing *AtMYB12* accumulate enhanced content of rutin and can be used as a potential alternative source of rutin as well as biopesticides against insect pests.

Keywords *AtMYB12* · Callus culture · Flavonols · Insect resistance · Rutin

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
ACTs	Acetyltransferases
ANS	Anthocyanidin synthase
CHS	Chalcone synthase
CHR	Chalcone reductase
CHI	Chalcone isomerase
C4H	Cinnamate 4-hydroxylase
4CL	4-Coumaroyl CoA ligase
DFR	Dihydroflavonol 4-reductase
F3H	Flavanone 3-hydroxylase
F3′H	Flavonoid 3′-hydroxylase
F3′5′H	Flavonoid 3′5′-hydroxylase
FLS	Flavonol synthase
GT	Glucosyltransferase
LAR	Leucoanthocyanidin reductase
LDOX	Leucoanthocyanidin dioxygenase
MS	Murashige and Skoog medium
OMT	*O*-methyltransferase
PAL	Phenylalanine ammonia lyase
RT	Rhamnosyltransferase

Introduction

Flavonoids are plant secondary metabolites with remarkable chemical diversity. On the basis of aglycone structure,

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flavonoids are categorized into flavanones, flavonols, isoflavones, proanthocyanidins and anthocyanidins (Marais et al. 2006; Ververidis et al. 2007). Due to the ubiquitous occurrence and manifold functions of flavonoids, including their role in plant pigmentation, the pathway leading to the flavonoid biosynthesis has been the focus of extensive research in diverse plant species (Grotewold 2006). Therefore, among plant secondary metabolism, flavonoid biosynthesis is probably the best studied pathway at biochemical and molecular levels (Koes et al. 2005; Lepiniec et al. 2006; Hichri et al. 2011). The pathway known as phenylpropanoid pathway (Fig. 1) has phenylalanine ammonia lyase (PAL) being the entry step enzyme (Ververidis et al. 2007). The downstream enzymatic activities lead to the formation of diverse aglycone flavonoid backbones, which are further decorated by various residues through modifying enzymes such as glycosyltransferases and acyltransferases (Grotewold 2006). Out of various flavonoids, flavonols such as quercetin, kaempferol and rutin have been implicated in protection of plants against damaging UV radiation (Winkel-Shirley 2002) and regulation of auxin transport (Peer and Murphy 2007). Flavonols also exhibit a range of health beneficial properties through providing protection against cardiovascular diseases, certain types of cancers, degenerative disorders (Korkina et al. 2007; Nijveldt et al. 2001), platelet aggregation (Graf et al. 2005) and osteoporosis (Trivedi et al. 2009).

Secondary plant products including flavonoids have also been shown to act as insect toxicant (Schultz 2002). Enhanced biosynthesis of the few secondary plant products through pathway engineering has been shown to develop insect resistance in plants (Kappers et al. 2005; Johnson and Dowd 2004; Johnson et al. 2007; Misra et al. 2010; Rodriguez et al. 2011). The flavonoids especially rutin present in extracts of selected wild-type soybean genotypes was shown to confer resistance against seed-sucking insects (Piubelli et al. 2005). Consumption of artificial diet containing leaf extracts of PI 227687, an insect resistant soybean cultivar, by insect larvae caused strong allelochemical effects, such as weight reduction and increased mortality (Smith and Fischer 1983). Investigations suggested that the fraction with insecticidal activity was composed of flavonoids especially rutin (Hoffmann-Campo et al. 2001). It has been demonstrated that rutin interacts as a non-competitive inhibitor with arginine kinase, a key enzyme in the cellular energy metabolism of insects (Wu et al. 2009), which might be one reason for its insecticidal activity. Recently, enhanced accumulation of rutin in tobacco through heterologous expression of *Arabidopsis* transcription factor, *AtMYB12*, has been shown to confer

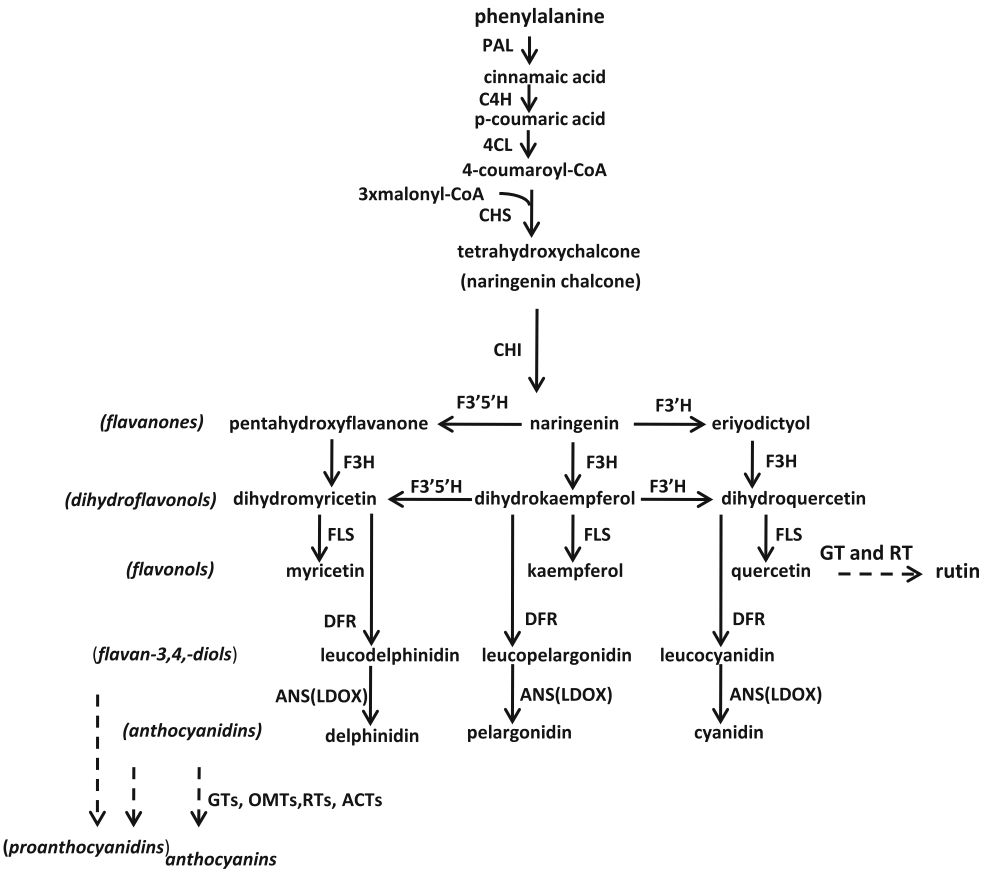

Fig. 1 Basic flavonoid biosynthesis pathway in plants. Different classes of flavonoids have been shown in *brackets* in front of their position in the pathway, e.g., flavonols includes quercetin, kaempferol and myricetin (aglycone forms) and rutin (glycosylated quercetin). *Dashed arrows* represent multiple enzymatic steps. *ACTs* acetyltransferases, *ANS* anthocyanidin synthase, *CHS* chalcone synthase, *CHR* chalcone reductase, *CHI* chalcone isomerase, *C4H* cinnamate 4-hydroxylase, *4CL* 4-coumaroyl CoA ligase, *DFR* dihydroflavonol 4-reductase, *F3H* flavanone 3-hydroxylase, *F3′H* flavonoid 3′-hydroxylase, *F3′5′H* flavonoid 3′5′-hydroxylase, *FLS* flavonol synthase, *GT* glucosyltransferase, *LAR* leucoanthocyanidin reductase, *LDOX* leucoanthocyanidin dioxygenase, *OMT* *O*-methyltransferase, *PAL* phenylalanine ammonia lyase, *RT* rhamnosyltransferase

resistance toward insect pests *Spodoptera litura* and *Helicoverpa armigera* (Misra et al. 2010).

The transcriptional regulation of structural genes of flavonoid pathway is a primary mode to regulate the biosynthesis of diverse classes of flavonoids under developmental, tissue-specific and environmental cues (Lepiniec et al. 2006; Hichri et al. 2011). Members of R2R3-MYB family of transcription factors have been demonstrated as transcriptional regulators of structural genes of flavonoid biosynthesis (Lepiniec et al. 2006). The R2R3-MYB transcription factors activate the expression of multiple structural genes of flavonoid pathway and therefore have been utilized for metabolic engineering of various classes of flavonoids, such as anthocyanin (Butelli et al. 2008), flavonols (Luo et al. 2008; Misra et al. 2010) and proanthocyanidins (Xie et al. 2006). Earlier, Misra et al. (2010) and Luo et al. (2008) have shown that the constitutive expression of *AtMYB12* in tobacco leads to the highly enhanced accumulation of flavonols including rutin. In addition, using an integrated metabolomic and transcriptomic approaches, it was established that the *AtMYB12* expression affects a variety of pathways leading to the flux availability for the flavonol biosynthesis in transgenic tobacco (Misra et al. 2010). As plant cell cultures offer an excellent system for large-scale production of various secondary metabolites due to rapid biomass production (Matkowski 2008; Smetanska 2008), in this study, callus cultures of wild-type (WT) and *AtMYB12*-expressing transgenic tobacco line accumulating enhanced rutin content have been developed. The effect of light and nutrient nitrogen status on modulation of rutin content has also been studied to demonstrate the regulation of rutin biosynthesis in callus culture by these factors. Furthermore, study demonstrates several fold enhanced content of rutin in transgenic callus responsible for the mortality of the larva of two of the insect pests, *S. litura* and *H. armigera*.

Materials and methods

Plant materials

Leaves from *AtMYB12*-expressing transgenic (*Nicotiana tabacum* cv. Petit Havana) and WT tobacco (cv. Petit Havana) plants were used for callus induction. *AtMYB12*-expressing homozygous transgenic tobacco lines were developed and characterized earlier (Misra et al. 2010). These transgenic lines displayed enhanced expression of genes related to flavonoid biosynthesis pathway and accumulated increased rutin level. Out of all the lines studied, line 7 (T7)-accumulated maximum rutin has been used in this study. The tobacco plants were grown in vitro on half-strength MS medium (Murashige and Skoog 1962) after surface sterilization of seeds with NaOCl. The leaves of 4-week-old seedlings were used for callus induction.

Callus induction

Young leaves from tobacco seedlings were aseptically cut into pieces of 1 cm^2 using a sterile razor blade. The leaf pieces were inoculated (adaxial side facing up) onto the callus induction medium (modified MS medium with Gamborg's B5 vitamins, 0.1 mg/L kinetin and 0.5 mg/L 2,4-D) in Petri plates. Antibiotics (kanamycin, 100 mg/L) was used for callus induction of transgenic lines and removed once calluses had been induced for further experiments. The plates were incubated in culture room under the conditions of 16 h of light (100 $\mu E/m^2/s$, Phillips, India) and 8 h of dark at 25 ± 1 °C. The induced calluses from the WT and transgenic tobacco were repeatedly subcultured over aforementioned callus induction medium at 3-week intervals. After 2 months of callus induction, various experiments were performed.

Growth kinetics study

Twenty-day-old calluses were used for subculturing and growth kinetic studies. Weighed amount of calluses (1 g of fresh weight) were inoculated in Petri plates with callus induction medium (25 mL). The calluses were harvested and weighed at 5-day intervals until 25 days with six independent replicates for each time point ($n = 18$). The growth index was calculated using the formula: $F_w - I_w / I_w$, where I_w and F_w are the initial (at the time of inoculation) and final weight (after harvesting) of callus, respectively. The harvested calluses were frozen in liquid nitrogen and kept in −80 °C freezer until further use.

Dark treatment

One gram fresh weight of 20-day-old calluses were inoculated onto the callus induction medium and the cultures were kept in culture room with similar growth conditions, except for the one set of cultures that was grown in the dark. The calluses were harvested and weighed after 20 days of in vitro growth. After calculation of growth index, the calluses were frozen in liquid nitrogen and kept in −80 °C freezer until further use. The data was analysed using six replicates each from three independent experiments ($n = 18$).

Nitrogen treatment

Weighed amount (1 g) of 20-day-old calluses were inoculated in four different (N1, N2, N3 and N4) callus induction media. These media were similar to aforementioned callus

induction medium in every respect except for the concentration of nitrogen source. In MS medium, the concentrations of two nitrogen sources, NH_4NO_3 and KNO_3, are 20 and 18.8 mM, respectively. The nutrient nitrogen concentrations were kept lower in N1 (0 mM NH_4NO_3 + 18.8 mM KNO_3) followed by N2 (20 mM NH_4NO_3 + 0 mM KNO_3) and N3 medium (10 mM NH_4NO_3 + 9.4 mM KNO_3) in comparison to full MS salt-containing callus induction medium (N4). After 20 days of in vitro growth, calluses were harvested and growth index was calculated in each case. The experiment was performed using six replicates each in three independent experiments ($n = 18$).

Gene expression analysis

Total RNA was isolated from the 20-day-old calluses (Asif et al. 2006) and subsequently treated with RNase-free DNase (Fermentas, Life Sciences, Ontario, Canada). RNA was subjected to reverse transcription to generate first strand cDNA using oligo dT primers (MBI, Fermentas). PCR was carried out using first strand cDNA and gene-specific primers as described earlier by Misra et al. (2010). The sequences of the various oligonucleotides used are given in Table 1.

Analysis of flavonols

For the estimation of flavonols, fresh tissue (2 g) was ground into fine powder in liquid N_2 and extracted with methanol:water (70:30) overnight at room temperature with rotation (150 rpm). Extracts were filtered through a 0.2 μm filter (Millipore) before separation and quantitation by liquid chromatography as described earlier (Pandey et al. 2012). The extract of calluses were used for the separation of qualitative and quantitative analysis of flavonoids by HPLC–PDA with a Shimadzu (Japan) LC-10 system comprising an LC-10AT dual pump system, an SPD-M20A PDA detector and rheodyne injection valve furnished with a 20 μL sample loop. Compounds were separated on a 5 μm pore size Merck RP-C18 column (250 mm length and 4.6 mm internal diameter) protected with guard column of same chemistry. The mobile phase was a gradient prepared from 0.5 % (v/v) phosphoric acid in HPLC-grade water (component A) and methanol (component B). Before use, the solvents were filtered through 0.45 μm nylon filters and de-aerated in an ultrasonic bath.

Analysis of the flavonols was carried out as described by Niranjan et al. (2011) with modifications (Pandey et al. 2012). Elution was carried out at a flow rate of 0.8 mL/min with 0.5 % phosphoric acid as solvent A and methanol as solvent B using a gradient elution in 0–5 min with 75–70 % A, 5–10 min with 70–50 % A, 10–15 min with 50–20 % A and 15–25 min with 20–80 % A. Each time the column was equilibrated by running the mobile phase and post-run program by injecting the methanol as blank.

Insect bioassay

Insect bioassay was carried out against neonate larvae of *S. litura* and *H. armigera* as described earlier (Misra et al. 2010). Insect cultures continuously reared in the laboratory for more than 2 years (*H. armigera* on semi-synthetic diet and *S. litura* on *Ricinus communis* leaves) were used for the study. Insect cultures were maintained in the laboratory at a constant temperature of 26 ± 2 °C and 70 % relative humidity, and the bioassay was also carried out in similar condition. Ethanol extract of WT and transgenic callus and purified rutin dissolved in ethanol were mixed separately with semi-synthetic diet (2 mg/g diet) before solidifying. Ethanol was used in the extraction for insect bioassay as methanol causes toxicity to the larvae (Liu et al. 2003) when mixed with semi-synthetic diet. Extraction procedure by ethanol is similar to methanol extraction of flavonols. Semi-synthetic diet mixed with similar volume of ethanol served as control. Ethanol was evaporated from the media poured in Petri plates (60 mm) by keeping them open for 4 h. Though methanol and ethanol both evaporate, however, there is possibility of traces of solvents remaining in the diet. Since methanol has been reported toxic to the

Table 1 The primers used for semi-quantitative PCR analysis of selected genes

S. no.	Gene name	Forward primer	Reverse primer
1.	UBI	5′-TCCAGGACAAGGAGGGTAT-3′	5′-GAGACCTCAGTAGACAAAGC-3′
2.	AtMYB12	5′-AACCAAGGGAATCTCGACTGTCT-3′	5′-CGTCGTCATGATCTAACGGTTC-3′
3.	PAL	5′-GCCAACAGGATAAAAGAATGC-3′	5′-CAAGAACATATAAGCACATTG-3′
4.	CHS	5′-CCTTTGGGAATTTCTGATTGG-3′	5′-TCCCACAATATAAGCCCAAGC-3′
5.	F3H	5′-AGCTAGAGACTACTCCAGGTG-3′	5′-AACCGTGATCCAAGTTTTGCCA-3′
6.	CHI	5′-GACGGGTAAGCAATACTCAGAGAAG-3′	5′-AACTAGACTCCAATTTCTGGAATG-3′
7.	FLS	5′-AAGTGATAAATCATGGAATTCCAG-3′	5′-TCTTCACCACCTGCTGCCTCCATC-3′

insects (Liu et al. 2003), we preferred ethanol extraction for insect bioassay. Neonate larvae of *S. litura* (10 larvae/replication) released on diet and three replications ($n = 30$) were maintained for each treatment. In the case of *H. armigera*, the larvae (30 nos.) were released individually on diet to avoid cannibalism. Larval mortality and the weight of surviving larvae were recorded after 5 and 7 days of treatment.

Statistical analysis

All experiments were repeated three times with at least three replicates per treatment. The data were analyzed by Student's paired t test, and the mean values under each treatment were compared at $P \leq 0.05$–0.001.

Results

Establishment and growth of callus cultures

In order to develop callus cultures, leaf explants from WT and *AtMYB12*-expressing transgenic plants (T7 line; Misra et al. 2010) were incubated over callus induction medium containing kinetin and 2,4-D. After 20 days of incubation, calluses were induced from wounded sites of the cultured tobacco leaf explants. The calluses from both WT and transgenic plants had soft and friable texture. These calluses were repeatedly subcultured on the same medium at 3-week intervals. At the time of subculturing and air exposure, browning was evident in all transgenic tobacco calluses, while no such browning was noted in WT calluses (Fig. 2a). However, after 1 week of growth over callus induction medium, such color demarcation did not persist and both WT and transgenic calluses appeared pale yellow and were indistinguishable from each other. The growth kinetics of the calluses was studied up to 25 days of in vitro growth by scoring growth index at various time points (Fig. 2b). WT and transgenic calluses followed similar growth pattern. For both the calluses, an initial slower growth up to 10 days followed by rapid growth up to 20 days, and thereafter an apparent stationary phase up to 25 days was observed. Hence, the tobacco calluses appeared to follow a roughly sigmoid growth pattern.

Flavonol content in callus cultures and gene expression analysis

Earlier studies suggest that flavonol content of leaves of transgenic tobacco lines expressing *AtMYB12* was several folds higher than that of WT tobacco leaves (Luo et al. 2008; Misra et al. 2010). A HPLC-based analysis of flavonol levels in callus cultures was carried out using

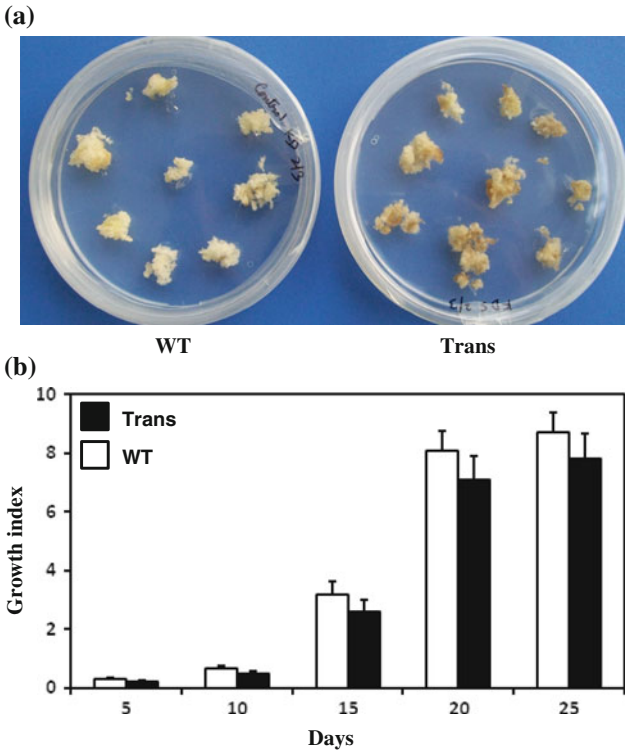

Fig. 2 Establishment and growth of transgenic and WT tobacco calluses. Color demarcation between WT and transgenic calluses just after subculturing over callus induction medium (**a**). Growth kinetic study of WT and transgenic calluses (**b**). Weighed amount of calluses were inoculated in callus induction medium followed by harvesting at different time points (0, 5, 10, 15 and 25 days). The growth index was calculated at each time points. Data are expressed as mean ± SD of three independent experiments, each experiment consisting of six replicates ($n = 18$)

methanolic extracts of callus tissue from varying time points of in vitro growth. The analysis suggested rutin as the predominant flavonol in both WT and transgenic calluses (Fig. 3a, c). Trace amounts of quercetin and kaempferol could also be detected in a few samples from transgenic calluses (data not shown). At each time point of in vitro growth, the rutin content of transgenic callus was several folds higher than that of WT tobacco, e.g., at 20th day of in vitro growth, the rutin content of transgenic callus was 0.52 ± 0.05 mg/g of fresh weight, which was about 34-fold more than that of the WT callus. A similar fold change in rutin content has been previously reported in the case of *AtMYB12*-expressing transgenic tobacco plants (Misra et al. 2010). The analysis also indicates non-significant variation in the rutin content of callus at various time points of in vitro growth.

The expression of genes involved in flavonol biosynthesis was assessed in callus cultures through semi-quantitative RT-PCR. We observed up-regulation of genes, such as *PAL*, *CHS*, *CHI*, *F3H* and *FLS*, in the transgenic calluses compared to the WT calluses (Fig. 3b). These results

suggested that transgenic calluses derived from *AtMYB12*-expressing tobacco have inherent potential of biosynthesizing higher levels of rutin.

Effect of light on growth and rutin content of callus

The flavonoid biosynthesis in plants is influenced by various environmental factors including light (Cominelli et al. 2008). To find out impact of light over in vitro flavonol biosynthesis and growth in tobacco callus cultures, one set of cultures was incubated in continuous dark and another set was subjected to 16 h light/8 h dark cycle. After 20 days of in vitro growth, significantly reduced growth index was observed for callus cultures grown in continuous darkness (Fig. 4a). HPLC analysis of methanolic extracts from various callus samples indicated that the rutin content of dark-grown transgenic callus was significantly reduced in comparison to the transgenic callus grown in 16 h light/8 h dark cycle (Fig. 4b and Supplementary Fig. S1). Furthermore, rutin could not be detected in WT tobacco callus grown in continuous darkness (Fig. 4b and Supplementary Fig. S1). These results suggest that there is a modulating effect exerted by light over flavonol biosynthesis in WT and transgenic calluses.

Effect of nitrogen deficiency on growth and rutin content of callus

Nitrogen deficiency is an important factor for the activation of anthocyanin and flavonol biosynthesis (Stewart et al. 2001; Lea et al. 2007). Therefore, the effect of nitrogen level over growth and rutin content of WT and transgenic tobacco calluses was studied by decreasing the concentration of nitrogen sources (NH_4NO_3 and KNO_3) in MS medium. After 20 days of in vitro growth over a range of modified callus induction media (N1, N2, N3 and N4), growth index of callus cultures was calculated. Low nitrogen concentration in the medium, N1, resulted in

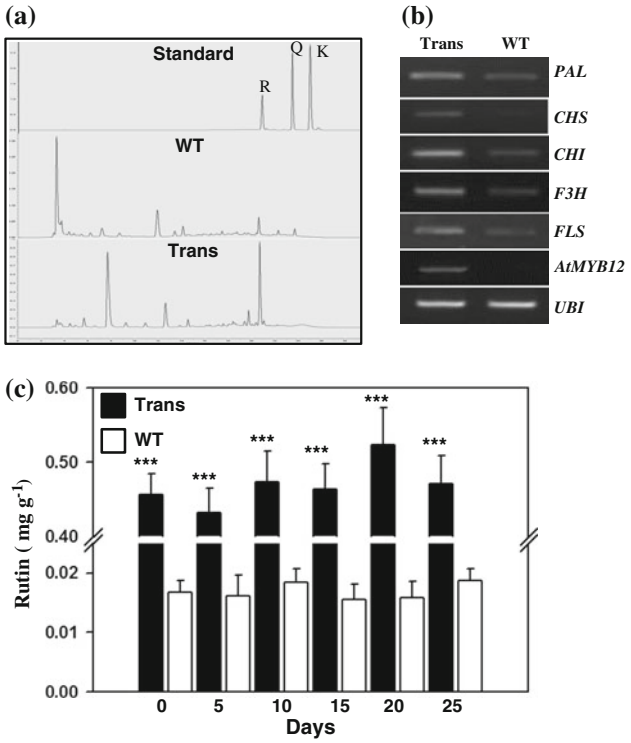

Fig. 3 Analysis of flavonols and gene expression in WT and transgenic calluses. Methanolic extracts from WT and transgenic calluses fractionated using HPLC (**a**) and quantified using standard (**c**). Expression of structural genes of flavonoid pathway leading to flavonol biosynthesis as well as *AtMYB12* was profiled using semi-quantitative RT-PCR of total RNA isolated from WT and transgenic calluses (**b**). Expression of ubiquitin has been taken as the internal control. The graph of rutin content (**c**) shows mean ± SD of three independent experiments, each experiment consisting of six replicates ($n = 18$). ***Significantly different at $P \leq 0.001$ according to Student's unpaired *t* test

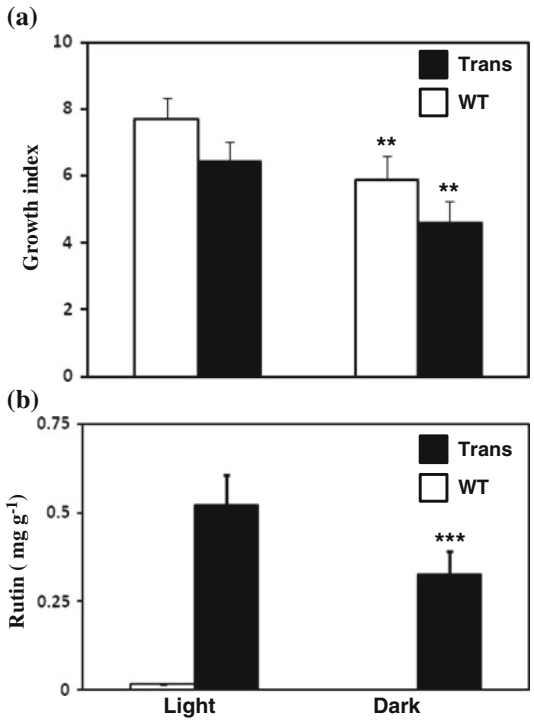

Fig. 4 Effect of light over growth and rutin content of tobacco calluses. One set of calluses was incubated in continuous darkness and another set was kept in 16 h light and 8 h dark conditions (mentioned as light). After 20 days of growth, WT and transgenic calluses from both sets were harvested and analyzed for growth index (**a**) and rutin content (**b**). A typical chromatogram of HPLC used for the analysis of rutin content is given in Supplementary Fig. S1. Data are expressed as mean ± SD of three independent experiments, each experiment consisting of six replicates ($n = 18$). ** and *** significantly different at $P \leq 0.01$ and 0.001, respectively, according to Student's unpaired *t* test

reduction in the growth index of both types of calluses as compared with the callus cultures grown over full nutrient nitrogen concentration (N4) (Fig. 5a). Furthermore, the callus cultures grown on medium lacking NH_4NO_3 exhibited the highest growth reduction. The estimation of flavonol level in WT and transgenic calluses grown over aforementioned medium suggested that the rutin content of WT calluses grown on nutrient-nitrogen-deficient media (N1, N2 and N3) was significantly higher than that of WT callus grown on full MS medium (N4) (Fig. 5b and Supplementary Fig. S2). The highest increase in rutin content was recorded in the case of WT calluses grown on NH_4NO_3-less callus induction medium (N1), e.g., rutin content of callus grown on N1 medium was 0.079 ± 0.012 mg/g of fresh weight, which was about fivefold higher than that of WT calluses grown on medium having full MS salts. However, the rutin content of transgenic calluses did not show any significant alteration following nitrogen deficiency in the growth medium.

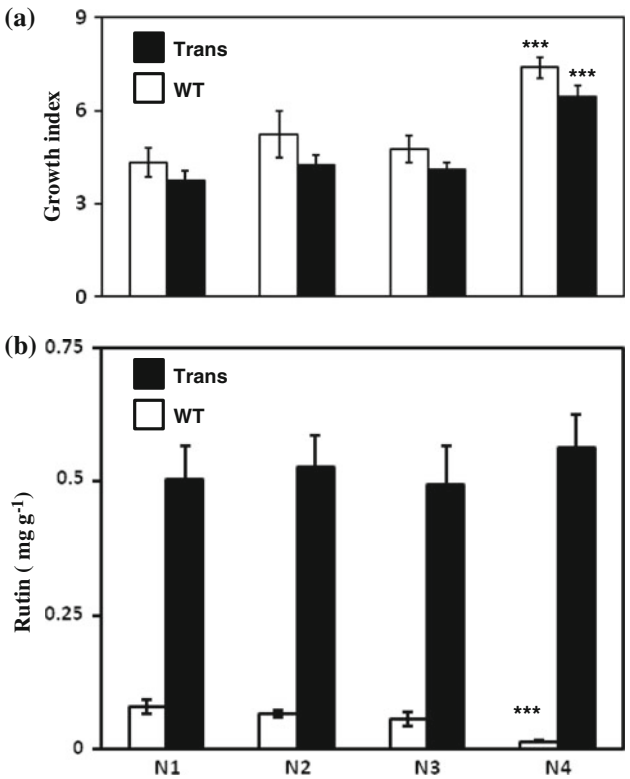

Fig. 5 Effect of nitrogen deficiency over growth (**a**) and rutin content (**b**) of WT and transgenic calluses. WT and transgenic calluses were grown over callus induction media (N1, N2 and N3) having lower concentrations of nitrogen sources as compared to full MS composition (N4). After 20 days of callus growth, growth index and flavonol content were analyzed. A typical chromatogram of HPLC used for the analysis of rutin content is given in Supplementary Fig. S2. Data are expressed as mean ± SD of three independent experiments, each experiment consisting of six replicates ($n = 18$). ***Significantly different at $P \leq 0.001$ according to Student's unpaired t test

Insect toxicity assay

In order to assess the insect toxicant potential of rutin in transgenic calluses, neonate larvae of *S. litura* and *H. armigera* were fed on synthetic diet mixed with ethanolic extract of WT and transgenic calluses as well as purified rutin from transgenic calluses. After 7 days, 20 % mortality as well as significant growth reduction in surviving insects (91.8 %) was reported in the case of *S. litura* larvae fed on synthetic diet with ethanolic extract of transgenic callus (Fig. 6a; Table 2). Besides, in the case of *H. armigera* larvae fed on the synthetic diet with transgenic calluses for 7 days, significant growth reduction (91.4 %) as well as mortality (40 %) were evident. However, around 20 % mortality as well as growth reduction (48.8 %) could also be scored in the case of synthetic diet with WT callus extract, though it was significantly lower than that of transgenic extract (Fig. 6b; Table 2). Significant growth reduction as well as mortality was also observed for both the insect species fed with synthetic diet containing rutin (2 mg/mL) isolated from transgenic calluses (Fig. 6; Table 2). It may be due to the fact that tobacco is not a natural host for *H. armigera*. These results clearly suggest that the callus grown from transgenic tobacco expressing AtMYB12 transcription factor can be a good source of rutin and could be used as antifeedant for insect pests to save agricultural crops.

Discussion

AtMYB12 is a transcriptional activator of the structural genes of the flavonol biosynthesis (Mehrtens et al. 2005; Stracke et al. 2007). The leaves of transgenic tobacco lines

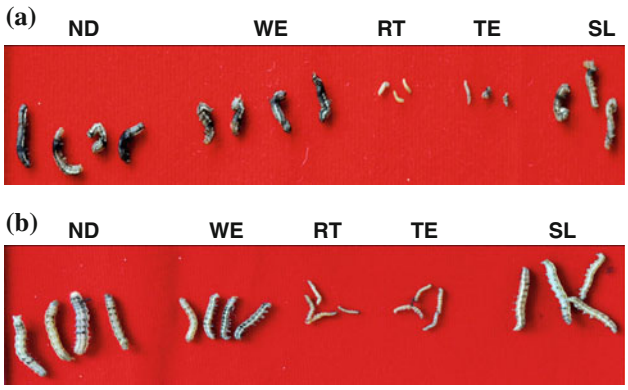

Fig. 6 Insect bioassay showing growth reduction of *S. litura* (**a**) and *H. armigera* larvae (**b**). Data were recorded after 7 days of release on semi-synthetic diet containing WT callus extract (WE, 10 mg/mL), rutin (RT, 2 mg/mL), transgenic callus extract (TE, 10 mg/mL) and solvent (SL). ND stands for the normal diet. In the case of *H. armigera*, the larvae were released individually to avoid cannibalism

Table 2 Effect on growth and mortality of larvae fed on semi-synthetic diet supplemented with different extracts and purified rutin

Treatment	After 5 days (mean ± SE)			After 7 days (mean ± SE)		
	Larval weight (mg)	Growth reduction (%)	Mortality (%)	Larval weight (mg)	Growth reduction (%)	Mortality (%)
Spodoptera litura						
Normal diet	6.7 ± 0.3^{d}	–	0.0	13.6 ± 1.1^{b}	–	0.0
WT ext.	5.7 ± 0.4^{c}	14.1	0.0	14.2 ± 1.3^{b}	−4.4	0.0
Transgenic ext.	0.7 ± 0.1^{a}	90.1	20 ± 0.0	1.1 ± 0.8^{a}	91.8	30.0 ± 5.0
Rutin	1.9 ± 0.2^{b}	71.7	0.0	1.7 ± 1.1^{a}	87.2	0.0
Ethanol	6.0 ± 0.3^{cd}	16.6	0.0	14.4 ± 11.3^{b}	−5.7	0.0
Helicoverpa armigera						
Normal diet	5.6 ± 0.7^{a}	0.0	0.0	27.8 ± 1.6^{c}	0.0	0.0
WT ext.	2.3 ± 0.2^{b}	59.5	0.0	14.2 ± 1.2^{b}	48.8	20.0 ± 0.0
Transgenic ext.	1.2 ± 0.1^{b}	77.8	40.0 ± 5.8	2.4 ± 0.4^{a}	91.4	40.0 ± 5.8
Rutin	1.6 ± 0.1^{b}	71.0	25.0 ± 2.9	2.4 ± 0.3^{a}	91.5	25.0 ± 2.9
Ethanol	6.3 ± 0.8^{a}	−12.0	0.0	27.0 ± 2.0^{c}	3.0	0.0

Data were acquired through three independent replicates after releasing neonate larvae (10 larvae in each replicates) on semi-synthetic diet ($n = 30$). Means (each insect species separately) were compared using Duncan's multiple range test ($P = 0.05$). Means in columns carrying same letter are not different

expressing *AtMYB12* gene were shown to accumulate significantly elevated levels of various flavonols (Luo et al. 2008; Misra et al. 2010). Since flavonols have gained the status of nutraceuticals, large-scale plant-based production of these compounds is desirable. Realizing the advantages of plant cell cultures for industrial production of secondary metabolites, herein, we explored the potential of *AtMYB12* transcription factor for the activation of flavonol biosynthesis in callus cultures of tobacco.

In our previous study, different AtMYB12-expressing homozygous tobacco transgenic lines accumulated rutin (up to 3.2 mg/g fresh weight), which was the most dominant flavonol in non-hydrolyzed methanolic extract (Misra et al. 2010). This increase was more than 40-fold in T7 line in comparison with the WT plants. In this study, we have used this transgenic line for developing callus culture. The transgenic callus derived from *AtMYB12*-expressing transgenic tobacco line displayed higher content of rutin as compared to the WT callus. Maximum amount of rutin in leaves of *AtMYB12*-expressing transgenic plants was 3.2 mg/g of fresh weight, which was 40-fold higher in comparison to WT plant. In the present study, rutin content of transgenic callus is 0.52 ± 0.05 mg/g of fresh weight, which is also 34-fold more than that of the WT callus. Though callus culture of transgenic lines have lower level of rutin content in comparison to transgenic leaves, callus cultures have advantages over the whole plants as far as the secondary metabolite production is concerned. The advantages include rapid growth, easy manipulation of conditions for scaling up biomass and enhanced accumulation of concerned metabolites (Rao and Ravishankar 2002). The browning of transgenic calluses during subculturing could be due to the oxidation of phenolics and therefore indicates higher polyphenol/flavonoid/flavonol content (Pource et al. 2007).

Light is one of the most important modulators of flavonoid biosynthesis and is prerequisite for the induction of structural genes of flavonoid pathway under normal growth conditions. In the present work, it is evident that light positively affects rutin biosynthesis in both WT and transgenic calluses. Continuous incubation of callus cultures in the dark completely eliminated rutin accumulation in WT calluses. In addition, there was a significant reduction in the rutin level of dark-exposed transgenic callus. In *Arabidopsis*, the structural genes for flavonol biosynthesis are activated by at least two different families of transcription factors, one including MYB transcription factors, namely, AtMYB12, AtMYB111 and AtMYB11 (Stracke et al. 2007) and other having a bZip family transcription factor HY5 (Oravecz et al. 2006). Of these, MYB transcription factors are functionally redundant and involved in tissue-specific flavonol biosynthesis regulation. The HY5 transcription factor regulates flavonoid biosynthesis in a light-dependent manner though expression of AtMYB12 is also light activated (Stracke et al. 2010). Therefore, in dark-incubated callus cultures, the reduced expression of structural genes of flavonol biosynthesis could be assumed due to the inactivity of tobacco homolog of *Arabidopsis* HY5 that may, in turn, be responsible for the reduction in rutin content.

Our results clearly indicate a significant increase in rutin content of the WT callus grown on nitrogen-deficient nutrient medium. Similarly, in *Arabidopsis*, anthocyanin and flavonol levels were shown to be up-regulated during nitrogen deficiency (Stewart et al. 2001; Lea et al. 2007).

The positive effect of nitrogen deficiency over anthocyanin biosynthesis is mediated by up-regulated expression of anthocyanin-specific R2R3-MYB transcription factors *PAP1* and *PAP2*. Although *CHS* expression has been reported to be up-regulated by nitrogen deficiency (Scheible et al. 2004), the precise molecular mechanism of nitrogen deficiency-induced elevation of flavonol content in *Arabidopsis* has not been worked out. However, a transcriptional level regulation of flavonol biosynthesis genes could be a likely mechanism.

Earlier, it was demonstrated that the enhanced rutin content in *AtMYB12*-expressing transgenic tobacco is responsible for the insect tolerance (Misra et al. 2010). Similarly, both *H. armigera* and *S. litura* fed on a diet containing transgenic callus extracts or purified rutin showed significant mortality and growth reduction (Fig. 6; Table 2). However, *H. armigera* larvae fed on a diet containing WT callus extracts also showed growth reduction and 20 % mortality. Since, tobacco is not a natural host for *H. armigera*, the observed toxicity may be due to various natural metabolites like nicotine present in tobacco.

Taken together, this study suggests that the *AtMYB12*-expressing transgenic tobacco calluses are able to accumulate one of the health beneficial flavonols rutin. Buckwheat accumulating 0.8–1.0 % of rutin on a dry weight basis has been proposed as a potential source of rutin (Kim et al. 2005). Hairy root cultures of buckwheat have been shown to produce rutin from 0.8 to 1.2 mg/g on a dry weight basis (Lee et al. 2007). In these report, rutin production is lower in comparison to our study (0.55 mg/g fresh weight basis). The transgenic tobacco callus cultures developed in this study could be a potential alternative source of rutin. Moreover, the callus as such or isolated rutin could be used as biopesticides against insect pests. In addition, production of other important flavonols such as quercetin and kaempferol can be engineered in such callus cultures by further manipulation of expression of other structural genes such as F3′H and genes that encode for glycosyltransferases. These callus cultures also appear to be a good system to study the molecular mechanism of modulation of flavonoid biosynthesis by various exogenous factors.

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References

Asif M, Trivedi PK, Solomos T, Tucker M (2006) Isolation of high quality RNA from apple fruit. J Agri Food Chem 54:5227–5229

Butelli E, Titta L, Giorgio M, Mock HP, Matros A, Peterek S, Schijlen EG, Hall RD, Bovy AG, Luo J, Martin C (2008) Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. Nat Biotechnol 26:1301–1308

Cominelli E, Gusmaroli G, Allegra D, Galbiati M, Wade HK, Jenkins GI, Tonelli C (2008) Expression analysis of anthocyanin regulatory genes in response to different light qualities in *Arabidopsis thaliana*. J Plant Physiol 165:886–889

Graf LC, Milbury PE, Blumberg JB (2005) Flavonols, flavones, flavanones, and human health: epidemiological evidence. J Med Food 8:281–290

Grotewold E (2006) The genetics and biochemistry of floral pigments. Annu Rev Plant Biol 57:761–780

Hichri I, Barrieu F, Bogs J, Kappel C, Delrot S, Lauvergeat V (2011) Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. J Exp Bot 62:2465–2483

Hoffmann-Campo CB, Harbone JB, Mcaffery JB (2001) Pre-ingestive and post-ingestive effects of soybean extracts and rutin on *Trichoplusia ni* growth. Entomol Exp Appl 98:181–194

Johnson ET, Dowd PF (2004) Differentially enhanced insect resistance, at a cost, in *Arabidopsis thaliana* constitutively expressing a transcription factor of defensive metabolites. J Agric Food Chem 52:5135–5138

Johnson ET, Berhow MA, Dowd PF (2007) Expression of a maize Myb transcription factor driven by a putative silk-specific promoter significantly enhances resistance to *Helicoverpa zea* in transgenic maize. J Agric Food Chem 55:2998–3003

Kappers IF, Aharoni A, Van Herpen Teun WJM, Luckerhoff Ludo LP, Dicke M, Bouwmeester HJ (2005) Genetic engineering of terpenoid metabolism attracts bodyguards to *Arabidopsis*. Science 309:2070–2072

Kim KH, Lee KW, Kim DY, Park HH, Kwon IB, Lee HJ (2005) Optimal recovery of high-purity rutin crystals from the whole plant of *Fagopyrum esculentum* Moench (buckwheat) by extraction, fractionation, and recrystallization. Bioresour Technol 96:1709–1712

Koes R, Verweij W, Quattrocchio F (2005) Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. Trends Plant Sci 10:236–242

Korkina LG, Mikhalchik E, Suprum MV, Pastore S, Dal Toso R (2007) Molecular mechanisms underlying wound healing and anti-inflammatory properties of naturally occurring biotechnologically produced phenylpropanoid glycosides. Cell Mol Biol 53:84–91

Lea US, Slimestad R, Smedvig P, Lillo C (2007) Nitrogen deficiency enhances expression of specific MYB and bHLH transcription factors and accumulation of end products in the flavonoid pathway. Planta 225:1245–1253

Lee SY, Cho SI, Park MH, Kim YK, Choi JE, Park SU (2007) Growth and rutin production in hairy root cultures of buckwheat (*Fagopyrum esculentum* M.). Prep Biochem Biotechnol 37:239–246

Lepiniec L, Debeaujon I, Routaboul JM, Baudry A, Pourcel L, Nesi N, Caboche M (2006) Genetics and biochemistry of seed flavonoids. Annu Rev Plant Biol 57:405–430

Liu XH, Pan H, Mazur P (2003) Permeation and toxicity of ethylene glycol and methanol in larvae of *Anopheles gambiae*. J Exp Biol 206:2221–2228

Luo J, Butelli E, Hill L, Parr A, Niggeweg R, Bailey P, Weisshaar B, Martin C (2008) *AtMYB12* regulates caffeoyl quinic acid and flavonol synthesis in tomato: expression in fruit results in very high levels of both types of polyphenols. Plant J 56:316–326

Marais JPJ, Deavours B, Dixon RA, Ferreira D (2006) The stereochemistry of flavonoids. In: Grotewold E (ed) The science of flavonoids. Springer, New York, pp 1–46

Matkowski A (2008) Plant in vitro culture for the production of antioxidants—a review. Biotechnol Adv 26:548–560

Mehrtens F, Kranz H, Bednarek P, Weisshaar B (2005) The *Arabidopsis* transcription factor *MYB12* is a flavonol-specific regulator of phenylpropanoid biosynthesis. Plant Physiol 138:1083–1096

Misra P, Pandey A, Tiwari M, Chandrashekar K, Sidhu OP, Asif MH, Chakrabarty D, Singh PK, Trivedi PK, Nath P, Tuli R (2010) Modulation of transcriptome and metabolome of tobacco by *Arabidopsis* transcription factor, *AtMYB12*, leads to insect resistance. Plant Physiol 152:2258–2268

Murashige T, Skoog FA (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Nijveldt RJ, Van Nood E, Van Hoorn DE, Boelens PG, Van Norren K, Van Leeuwen PA (2001) Flavonoids: a review of probable mechanisms of action and potential applications. Am J Clin Nutr 74:418–425

Niranjan A, Pandey A, Misra P, Trivedi PK, Lehri A, Amla DV (2011) Development and optimization of HPLC–PDA–MS–MS method for simultaneous quantification of three classes of flavonoids in legume seeds, vegetables, fruits and medicinal plants. J Liq Chromatogr Relat Technol 34:1729–1742

Oravecz A, Baumann A, Mate Z, Brzezinska A, Molinier J, Oakeley EJ, Adam E, Schafer E, Nagy F, Ulm R (2006) Constitutively photomorphogenic 1 is required for the UV-B response in *Arabidopsis*. Plant Cell 18:1975–1990

Pandey A, Niranjan A, Misra P, Lehri A, Tewari SK, Trivedi PK (2012) Simultaneous separation and quantification of targeted group of compounds in *Psoralea corylifolia* L. using HPLC–PDA–MS–MS. J Liq Chromatogr Relat Technol. doi:10.1080/10826076.2011.636854

Peer WA, Murphy AS (2007) Flavonoids and auxin transport: modulators or regulators? Trends Plant Sci 12:556–563

Piubelli GC, Hoffmann-Campo CB, Moscardi F, Miyakubo SH, de Oliveira MCN (2005) Are chemical compounds important for soybean resistance to *Anticarsia gemmatalis*? J Chem Ecol 31:1509–1525

Pource L, Routaboul JM, Cheynier V, Lepiniec L, Debeaujon I (2007) Flavonoid oxidation in plants: from biochemical properties to physiological functions. Trends Plant Sci 12:29–36

Rao SR, Ravishankar GA (2002) Plant cell cultures: chemical factories of secondary metabolites. Biotechnol Adv 20:101–153

Rodriguez A, San Andres V, Cervera M, Redondo A, Alquezar B, Shimada T, Gadea J, Rodrigo MJ, Zacarias L, Palou L, Lopez MM, Castanera P, Pena L (2011) Terpene down-regulation in orange reveals the role of fruit aromas in mediating interactions with insect herbivores and pathogens. Plant Physiol 156:793–802

Scheible WR, Morcuende R, Czechowski T, Fritz C, Osuna D, Palacios Rojas N, Schindelasch D, Thimm O, Udvardi MK, Stitt M (2004) Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of *Arabidopsis* in response to nitrogen. Plant Physiol 136:2483–2499

Schultz JC (2002) Biochemical ecology: how plants fight dirty. Nature 416:267

Smetanska I (2008) Production of secondary metabolites using plant cell cultures. Adv Biochem Eng Biotechnol 111:187–228

Smith CM, Fischer NH (1983) Chemical factors of insect resistant soybean genotype affecting growth and survival of the soybean looper. Entomol Exp Appl 33:343–345

Stewart AJ, Chapman W, Jenkins GI, Graham T, Martin T, Crozier A (2001) The effect of nitrogen and phosphorus deficiency on flavonol accumulation in plant tissue. Plant Cell Environ 24:1189–1197

Stracke R, Ishihara H, Huep G, Barsch A, Mehrtens F, Niehaus K, Weisshaar B (2007) Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling. Plant J 50:660–677

Stracke R, Favory JJ, Gruber H, Bartelniewoehner L, Bartels S, Binkert M, Funk M, Weisshaar B, Ulm R (2010) The *Arabidopsis* bZIP transcription factor HY5 regulates expression of the PFG1/MYB12 gene in response to light and ultraviolet-B radiation. Plant Cell Environ 33:88–103

Trivedi R, Kumar A, Gupta V, Kumar S, Nagar GK, Romero JR, Dwivedi AK, Chattopadhya N (2009) Effects of Egb 761 on bone mineral density, bone microstructure, and osteoblast function: possible roles of quercetin and kaempferol. Mol Cell Endocrinol 302:86–91

Ververidis F, Trantas E, Douglas C, Vollmer G, Kretzschmar G, Panopoulos N (2007) Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part I. Chemical diversity, impacts on plant biology and human health. Biotechnol J 2:1214–1234

Winkel-Shirley B (2002) Biosynthesis of flavonoids and effects of stress. Curr Opin Plant Biol 5:218–223

Wu XQ, Zhu WJ, Lu JR, Xia Y, Yang JM, Zou F, Wang XY (2009) The effect of rutin on arginine kinase: inhibition kinetics and thermodynamics merging with docking simulation. Int J Biol Macromol 44:149–155

Xie DY, Sharma SB, Wright E, Wang ZY, Dixon RA (2006) Metabolic engineering of proanthocyanidins through co-expression of anthocyanidin reductase and the PAP1 MYB transcription factor. Plant J 45:895–907