Regulation of polyphenols accumulation by combined overexpression/silencing key enzymes of phyenylpropanoid pathway

Junli Chang[†], Jie Luo[†], and Guangyuan He[*]

China-UK HUST-RRes Genetic Engineering and Genomics Joint Laboratory, Key Laboratory of Molecular Biophysics, Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology (HUST), Wuhan 430074, China
[†]These authors contributed equally to this work
*Correspondence address. Tel: +86-27-87792271; Fax: +86-27-87792272; E-mail: guangyuan.he@bbsrc.ac.uk; hegy@hust.edu.cn

There is a growing interest in the metabolic engineering of plant with increased desirable polyphenols such as chlorogenic acid (CGA) and rutin. In this study, the effects of overexpression of both phenylalanine ammonia lyase (AtPAL2), the first enzyme of the phenylpropanoid pathway, and hydroxycinnamoyl-CoA quinate:hydroxycinnamoyl transferase (NtHQT), the last enzyme of CGA biosynthesis, and the overexpression of AtPAL2 together with silencing of NtHQT were investigated in tobacco. Transgenic tobacco plants overexpressing *AtPAL2* showed two and five times increases of CGA and rutin levels than the wild-type (WT) plants, respectively. Overexpression of NtHQT further increases the accumulation of CGA in the AtPAL2 plants to about three times than that of the WT level, while silencing of NtHQT in AtPAL2 plants results in ∼12 times increase in rutin level than that of the WT plants. Simultaneous overexpression of phenylalanine ammonia lyase (PAL) and overexpression/silencing HQT could be used for the production of functional food with increased polyphenols.

Keywords phenylalanine ammonia lyase; hydroxycinnamoyl-CoA quinate:hydroxycinnamoyl transferase; phenylpropanoid; metabolic engineering

Received: September 8, 2008   Accepted: November 19, 2008

Introduction

The phenylpropanoid pathway (**Fig. 1**) is responsible for the synthesis of a large range of low molecular weight polyphenolics that occur naturally in plant tissues, including flavonols, flavones, flavanones, flavanones, catechins, anthocyanins, isoflavonoids, dihydroflavonols, and stilbenes [1]. To date, more than 4000 flavonoids have been identified. As a group, flavonoids are involved in many aspects of plant growth and development, such as pathogen resistance, pigment production, UV light protection, pollen growth, and seed coat development [2]. There is increasing evidence to suggest that flavonoids are health-protecting components in the human diet as a result of their high antioxidant capacity [3–6]. This is also supported by their ability to induce human protective enzyme systems [7–9], and their protection against major diseases such as cardiovascular diseases, cancer [10], and age-related diseases such as dementia [11]. In addition, several epidemiological studies have suggested a direct relationship between cardioprotection and consumption of flavonols from dietary sources [12].

Chlorogenic acid (CGA) is the major soluble polyphenol in a lot of plants such as tobacco, tomato, and potato. It also accumulates to substantial levels in apples, pears, and coffee. Inhibition of CGA accumulation in tobacco results in accelerated cell death in mature leaves, typical of oxidative stress, and increased levels of the oxidized lipid malondialdehyde [13]. Because of its high bioavailability, CGA is probably more accessible than that of many other flavonoids as a potential antioxidant from plants. CGA can also limit low-density lipid oxidation, the major determinant of the initial events in atherosclerosis. Furthermore, it removes particularly toxic reactive substances by scavenging alkylperoxyl radicals and may prevent carcinogenesis by reducing the DNA damage they cause [14]. Recent research also suggested the responsibility of CGA for the reduction of diabetes [15]. CGA is therefore an important and yet somewhat overlooked dietary bioactive.

Another major and important polyphenol in plants is rutin (quercetin-3-*O*-rutinoside), which widely exists in
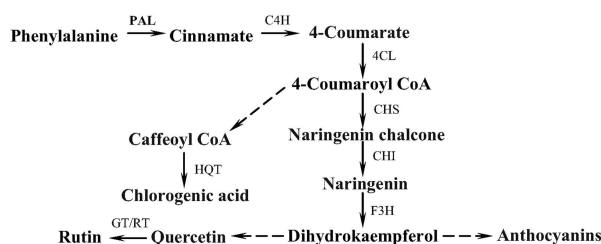
Fig. 1 Schematic diagram of part of the phenylpropanoid biosynthesis pathway Relative enzymes are indicated alongside the appropriate part of the pathway. Continuous arrow, single step; dashed arrow, multiple steps. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; HQT, hydrocycinnamoyl-CoA quintae transferase; GT/RT, glucosyl transferase/rhamnosyl transferase.

many crops and fruits. Lopez-Lopez et al. [16] reported that quercetin had the ability to reduce blood pressure and endothelial dysfunction in animal models of hypertension. The hypertension and oxidative stress prevention, and the vascular protection effects by chronic treatment of quercetin were also reported [17].

Based on these types of studies, there is growing interest in strategies to increase the polyphenol levels such as CGA and rutin may have potential to develop functional foods which enriched with health-protective polyphenols.

Phenylalanine ammonia lyase (PAL; E.C. 4.3.1.5) catalyses the first step of the phenylpropanoid pathway, which converts L-phenylalanine to trans-cinnamic acid. This enzyme has been suggested to be the key enzyme that controls the flux into the phenylpropanoid biosynthesis [18]. Different isoforms of PALs have also been suggested to be responsible for different branches of phenylpropanoid pathway. Hydroxycinnamoyl-CoA quinate:hydroxycinnamoyl transferase (NtHQT) catalyses the last step of CGA biosynthesis (**Fig. 1**) and proved to be critical for CGA accumulation in tomato [19].

In this paper, the effects of overexpression of both AtPAL2 and NtHQT, and overexpression of AtPAL2 and silencing of NtHQT on the accumulation of different phenylpropanoids in tobacco were investigated. Transgenic plants with increased PAL activities had higher accumulations of both CGA and rutin, which led to higher antioxidant capacity and enhanced tolerance to oxidative damage. The accumulation of CGA was even higher when NtHQT was overexpressed in the presence of AtPAL2, whereas decreased accumulation of CGA together with increased accumulation of rutin was achieved when NtHQT was silenced in the presence of

AtPAL2 than in the plants that only overexpressing AtPAL2. This strategy can be used for the development of functional foods that offer protection against cardiovascular disease and cancer through diet.

Materials and Methods

Materials
Nicotiana tobacum Samsun NN, Arabidopsis thaliana (Columbia ecotype), and Agrobacterium tumefaciens LBA4404 were used in this study.

AtPAL2 overexpression vector construction and transformation
Total RNA was extracted using the TRI-REAGENT (Sigma-Aldrich, St Louis, MO, USA) from flowers of A. thaliana (Columbia ecotype) plants according to the procedures provided by the manufacturer. First-strand cDNAs were synthesized by reverse transcription kit (TaKaRa, Dalian, China), and open reading frame (ORF) of AtPAL2 was amplified using primers P1: 5′-attB1-CCACCATGGATCAAATCGAAGCAATGTTG-3′ and P2: 5′-attB2-TAGCAAATCGGAATCGGAGCTG-3′ and cloned into the Gateway Donor vector (pDONR207) (Invitrogen, Carisbad, CA, USA) by BP reaction. The resulting vector pDONR207-AtPAL2 was then used to do the LR reaction to put AtPAL2 into Gateway compatible destination vector to create the overexpression vector pJAM1502-AtPAL2. Similarly, the ORF of NtHQT was amplified from tobacco cDNA using primers P3: 5′-attB1-CCACCATGGGAAGTGAAAAAATGATGA3′ and P4: 5′-attB2-TCAAAATTCATACAAATACTTC-3′ and eventually into pJAM1502-NtHQT. The gene silencing plasmid pFRN-NtHQT for NtHQT silencing was described previously [19]. The resulting plasmids were verified and used for stable transformation of tobacco using A. tumefaciens strain LBA4404 by the method described previously [20]. The transformed plants were selected on kanamycin.

Northern blot analysis
Total RNA was extracted and purified from tobacco leaves according to the procedures provided by manufacturer as mentioned above (Sigma-Aldrich). The resulted RNA was then separated on denaturing agarose gels and transferred onto nylon membranes (GE Healthcare, Piscataway, NJ, USA) and hybridized to radioactive DNA probes as described previously [19].

Determination of enzyme activities

PAL activity was assayed using the method as described previously [21] with slight modification. Briefly, the assay was carried out in a reaction mixture containing 0.1 M Tris–HCl buffer (pH 8.5), 1 mM 2-mercaptoethanol, 50 mM phenylalanine, and 1 ml aliquot of enzyme. The reaction was carried out for 20 min at $40°C$ and the rate of formation of trans-cinnamic acid was taken as a measure of enzyme activity using increase of 0.01 A_{290} unite as 3.09 nmol of trans-cinnamic acid formed. Protein levels were determined by Bradford method using BSA as a standard [22]. The determination of HQT activity was carried out as described previously [19].

Measurement of anthocyanin

Anthocyanin was extracted from petals of control and transgenic tobacco plants and measured by the method of Martin et al. [23]. Petals from tobacco flowers were weighed, cut into small pieces, and then extracted overnight at $4°C$ in sealed bottles containing 10 ml of 97% methanol and 3% HCl. The absorbance was measured at 530 nm, and the anthocyanin content was calculated as 1 A_{530} unit= 33 mM anthocyanin [23].

HPLC analysis of soluble phenolics in plants leaves

Soluble phenolics were extracted and analyzed [20], and the amount of CGA and rutin was measured using CGA and rutin standard from Sigma-Aldrich.

Trolox equivalent antioxidant capacity assay

The standard Trolox equivalent antioxidant capacity (TEAC) assay described by van den Berg et al. [24] was used with minor modifications. This assay assesses the total radical scavenging capacity based on the ability of a compound to scavenge the stable ABTS radical (ABTS$^{\bullet}$) in 6 min. The bluegreen ABTS$^{\bullet}$ was produced through the reaction between 7 mm ABTS and 2.45 mm potassium persulfate in water. This solution was stored in dark for 12–16 h before use. The concentrated ABTS$^{\bullet}$ solution was diluted with phosphate-buffered saline, pH 7.4, to a final A_{734} of 0.70 ± 0.02 at $37°C$. Stock solution of trolox was prepared in ethanol. Ten microliters of leaf extracts was added to 990 μl ABTS$^{\bullet}$ solution and A_{734} was measured. This was compared with a blank where 10 μl of the solvent was added to 990 μl of the ABTS$^{\bullet}$ solution. The reduction in absorbance 6 min after addition of the antioxidant was determined. The TEAC of the antioxidant was calculated by relating this decrease in absorbance to that of a trolox

solution on a molar basis. The hydrophilic and hydrophobic TEAC were measured by water and acetone extraction of leaf samples, respectively.

Generation of crossed transgenic lines

To generate AtPAL2/NtHQT lines, AtPAL2 overexpression line P3 was crossed to NtHQT line HO3. F1 seeds of AtPAL2/NtHQT plants were sowed on Murashige and Skoog (MS) plates containing 100 μg/l kanamycin and screened for the incorporation of both 35S-AtPAL2 and 35S-NtHQT by PCR amplification of genomic DNAs. Seedlings showed positive results in both PCR amplifications (AtPAL2/NtHQT lines) were transferred into soil and used for polyphenol analyses. Similarly, AtPAL2/NtHQT lines were generated between AtPAL2 overexpression line P3 and NtHQT silencing line HS2, screened for 35S-AtPAL2 and CHSA-NtHQT insertion and then analyzed for polyphenols. AtPAL2 overexpression line P3 was backcrossed with the wild-type (WT) tobacco (Samsun NN), and F1 seedlings that were kanamycin resistant and positive for AtPAL2 insertion (AtPAL2 lines) were transferred into soil and used as control plants. For polyphenol analyses, leaf samples from 12 plants of each line were pooled and analyzed for their CGA and rutin contents by HPLC.

Results

Generation of transgenic plants

The vectors used for the generation of the transgenic lines in this study were shown in **Fig. 2(A)**. Tobacco (N. tobacum Samsun NN) was transformed with a binary vector (pJAM1502-AtPAL2) containing the Arabidopsis PAL2 gene under the control of the constitutive cauliflower mosaic virus (CaMV) double 35S promoter (see the Materials and methods section) vis Argobacterium-mediated transformation to get AtPAL2 overexpression lines [**Fig. 2(B,C)**]. Similarly, pJAM1502-NtHQT and pFRN-Nthqt were introduced into tobacco to obtain NtHQT overexpression [**Fig. 2(D,E)**] and NtHQT silencing [**Fig. 2(F,G)**] lines, respectively. More than 30 independent transformants for each constructs were produced. After confirming the insertion by PCR, the transcription of the all these plants were further detected for their transcription levels of AtPAL2 for AtPAL2 overexpression and NtHQT for NtHQT overexpression/silencing plants, respectively, by Northern blot [**Fig. 2(B,D,F)**]. **Figure 2(B)** showed that the WT tobacco had no hybridizing band under washing conditions used in this study. This suggests that sequence similarity of the endogenous
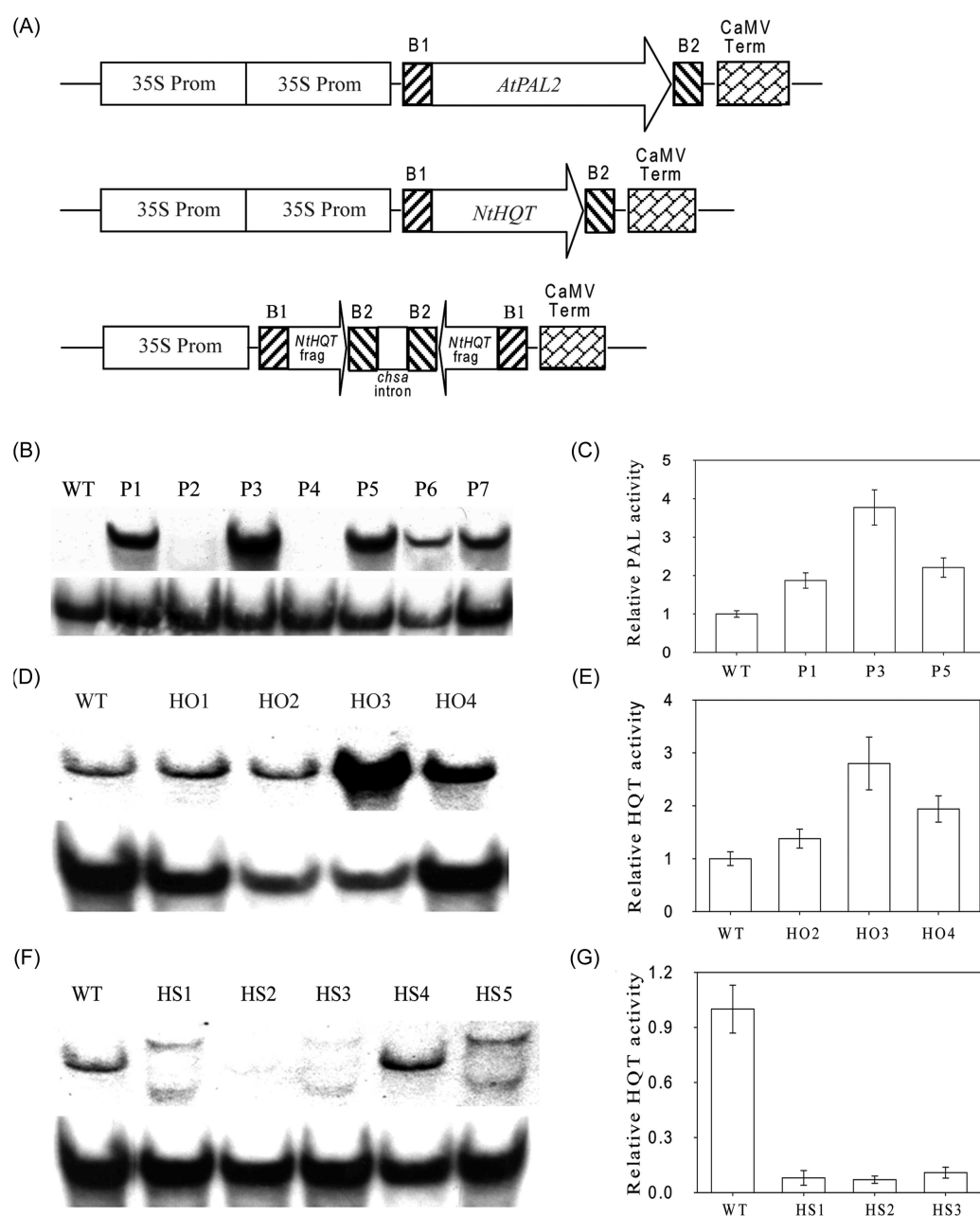
Fig. 2 Generation of *AtPAL2*, *NtHQT* overexpression and *NtHQT* silencing lines (A) Schematic representation of the constructs used in this study. Upper panel, *AtPAL2* overexpression; middle panel, *NtHQT* overexpression; lower panel, *NtHQT* silencing. 35S Prom, cauliflower mosaic virus 35S promoter; B1 and B2, attB1 and attB2 site of Gateway system, respectively; *AtPAL2*, *AtPAL2* coding region; *NtHQT*, *NtHQT* coding region; *NtHQT* frag, fragment of *NtHQT* coding region; CaMV Term, cauliflower mosaic virus (CaMV) terminator. (B) Northern blot analysis of total RNA from wild type (WT) and *AtPAL2* overexpression lines (P1–P7). (C) Relative PAL activities of WT and *AtPAL2* overexpression lines. (D) Northern blotting analysis of total RNA from WT and *NtHQT* overexpression lines (HO1–HO4). (E) Relative HQT activities of WT and *NtHQT* overexpression lines. (F) Northern blotting analysis of total RNA from WT and *NtHQT* silencing lines (HS1–HS4). (G) Relative HQT activities of WT and *NtHQT* silencing lines. Total RNA was fractionated, transferred to nylon membrane, and was hybridized to *AtPAL2* and *NtHQT* gene fragment, respectively. In the lower panel, the same blot was stripped and hybridized to ubiquitin probe to indicate the amount of RNA used in each sample. Leaf samples were taken at 45–50 days after sowing. Each value represents the mean ± SD.

tobacco gene to the *Arabidopsis* gene is relatively low and that any bands detected in the *AtPAL2* transgenic plants are due to the presence of the *Arabidopsis* gene only. The

target fragments were obtained from most of the transgenic plants detected by PCR amplification but not the control plant. We could not detect any *AtPAL2* expression

in lines P2 and P4. This might because the level of *AtPAL2* expression in this line was too low to be detected by Northern blot, or because of the silencing of the foreign gene after integration. To confirm the equal loading of RNA samples, RNA gel blot were also hybridized with a probe encoding the ubiquitin from tobacco. As shown in **Fig. 2(D)**, lines HO3, HO4, and HO2 of the NtHQT overexpression plants had increased *NtHQT* transcription levels than that of the WT plants, indicating the overexpression of *NtHQT* in these lines. In contrast, decreased *NtHQT* transcriptions were detected among most of the *NtHQT* silencing lines tested with HS2 and HS3 showed the lowest transcription levels of *NtHQT* [**Fig. 2(F)**]. To further confirm the above transgenic lines at the protein level, three independent lines that showed the highest overexpression/silencing effects were used for further enzyme activity assays. The leaves of the *AtPAL2* overexpression (lines P1, P3, and P5), *NtHQT* overexpression (lines HO2, HO3, and HO4), and *NtHQT* silencing (lines HS1, HS2, and HS3) plants were determined for their PAL or HQT enzyme activities, and the results are given in **Fig. 2(C,E,G)**, respectively. It could be seen that the PAL activities were correlated with the expressions of *AtPAL2* with the highest PAL activity obtained from leaves of P3, which was more than 3.5 times over the control levels. The highest *NtHQT* overexpression lines HO3 and HO4 had ~2.8 and 2 times activities than that of the control, whereas the remaining HQT activities in the *NtHQT* silencing lines HS1 to HS3 were ~10% (7% to 10%) of the control plants, respectively.

Overexpression of *AtPAL2* led to the increase of both CGA and rutin in tobacco leaves

The transgenic tobacco plants overexpressing *AtPAL2* had normal visual phenotype, growth characteristics, and fertility compared with the WT plants. However, when leaves of control and transgenic plants were extracted (with 0.1 M Tris–HCl buffer, pH 7.0) and clarified (by centrifuge and filtration), great difference could be seen as the brown coloration of the *AtPAL2* filtrate opposed to the translucent pale yellow color of the control [**Fig. 3(A)**]. This suggested the increase of the phenolic compounds in the transgenic plants than that in the control (the yellow-brown color was due to a mixture of oxidized phenolics that present in tobacco leaves). Three independent primary transformants P3, P5, and P1 that had the highest levels of transgene expression were used for further metabolite analyses. Non-hydrolyzed extracts were analyzed to determine the flavonoid contents in WT and the three independent primary *AtPAL2*

transformants. **Figure 3(B)** showed HPLC chromatograms obtained with non-hydrolyzed leaf extracts from control and the typical *AtPAL2* overexpressing tobacco. The major peaks in the extracts were identified to be CGA, cryptochlorogenic acid (3-caffeoyl quinic acid, 3-CQA), and rutin (quercetin rutinoside) by comparing the retention time and the UV spectra of the peaks of the extracts with the genuine standards and further confirmed by analyzing their fragmentation patterns using LC/MS/MS (data not shown). It could be seen from **Fig. 3(B)** that CGA and its isomer 3-CQA, with a retention time (RT) of 24.7 and 18.9 min, respectively, represented the major phenols in leaf of control and pJAM1502-AtPAL2 transformed tobacco. Significant amounts of rutin (RT = 34.4 min) also accumulated in the leaf of *AtPAL2* overexpression plants. Quantification of the major peaks revealed mean levels of up to 30.2 mg CGA and 2.8 mg rutin/g dry weight (DW) were obtained in non-hydrolyzed leaf extracts of *AtPAL2* overexpressing tobacco, representing increases of 2.1- and 4.7-fold over control levels, respectively [**Fig. 3(C)**]. In addition, the levels of CGA and rutin were in accordance with the expression level of the transgene. Therefore, we conclude that the profile change of the transgenic plants depended on the relative levels of expression of the introduced *AtPAL2* gene. One interesting thing to note was that there were no big difference in 3-CQA levels between control and transgenic tobacco, which might suggest that different enzymes might be responsible for the biosynthesis of different isomers of CGA.

The effect of *AtPAL2* overexpression on antioxidant capacity was determined by TEAC assay, and the results are shown in **Fig. 3(D)**. Hydrophilic TEAC increased with the increased *AtPAL2* overexpression. The highest hydrophilic TEAC of 23.6 μmol Trolox/g DW was obtained in *AtPAL2* overexpression line P3, which was ~2.5 times that of the control level. In contrast to the hydrophilic TEAC, no increase of hydrophobic TEAC was seen between the control and the transgenic plants, suggesting that the major effects of *AtPAL2* overexpression were restricted to the increase of hydrophilic substrates such as simple phenolics or flavonoids, with no effect on hydrophobic compounds (e.g. carotenoids, tocopherol). Measurement of anthocyanins extracted from tobacco flowers showed that control and transgenic plants had similar anthocyanin contents in their flowers [**Fig. 3(E)**], suggesting that metabolic flux in the anthocyanin biosynthesis pathway was not affected by the introduction of *AtPAL2*.

The above results showed that overexpression of *AtPAL2* could direct the flux into different branches of
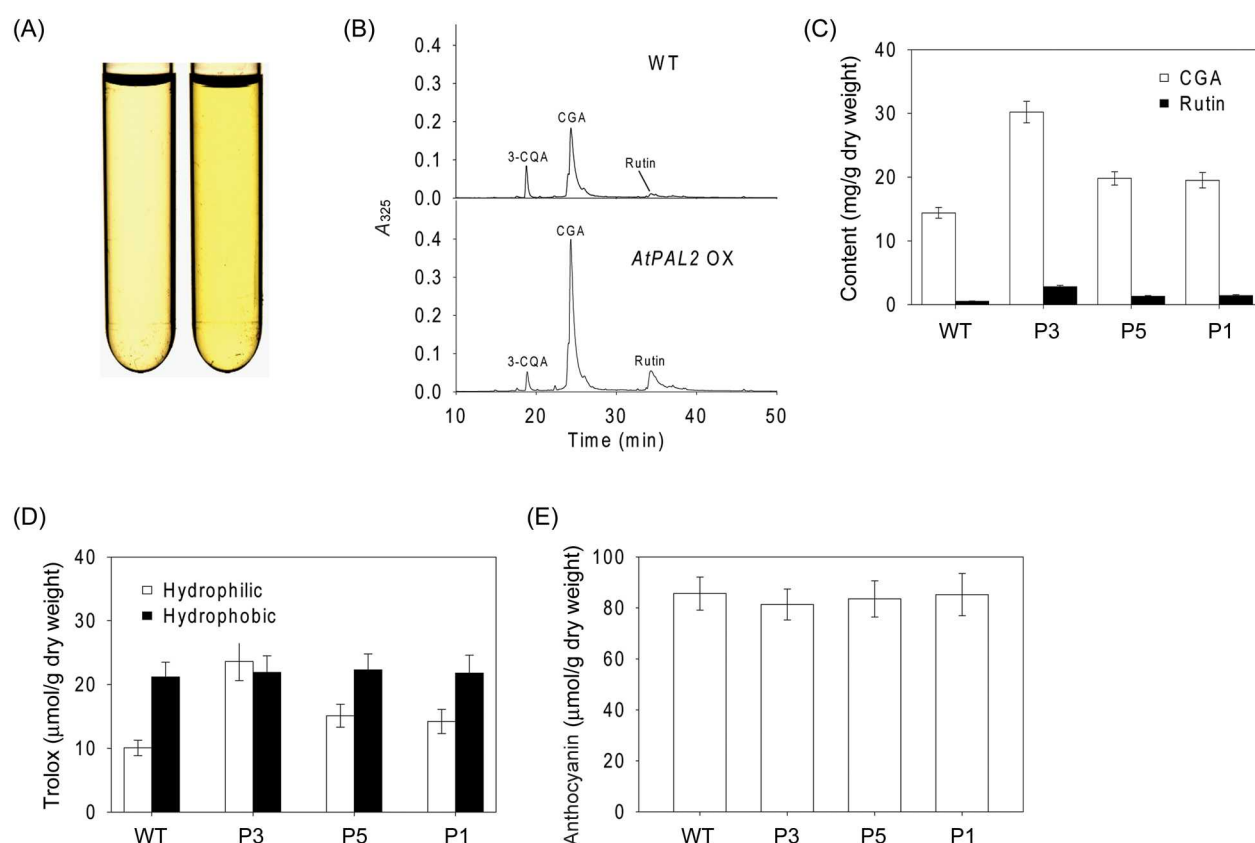
Fig. 3 Phenotype of *AtPAL2* overexpression tobacco lines   (A) Pictures of extracts of the soluble polyphenols in the wild type (WT) (left) and *AtPAL2* (right) overexpression tobacco leaves. (B) HPLC analysis of soluble phenolics in leaves of WT and typical *AtPAL2* overexpression tobacco line. 3-CQA, 3-caffeoyl quinic acid; CGA, chlorogenic acid. (C) Quantification of the major polyphenols in WT and *AtPAL2* overexpression tobacco leaves. (D) Antioxidant capacities in the WT and *AtPAL2* overexpression tobacco lines. Both hydrophilic and hydrophobic of the Trolox equivalent antioxidant capacity (TEAC) were measured. (E) Anthocyanin contents in the WT and *AtPAL2* overexpression tobacco lines. Leaf samples were taken at 45–50 days after sowing. Each value represents the mean ± SD.

the phenylpropanoid pathway and resulted in increased accumulation of both CGA and rutin in tobacco leaves.

Overexpression of *NtHQT* further increased CGA production in *AtPAL2* plants

To investigate whether the production of CGA can be further increased, tobacco plants harboring *AtPAL2* were crossed with plants overexpressing *NtHQT* (line P3 crossed with line H3, see the Materials and methods section). **Figure 4** showed that CGA accumulation in the *AtPAL2/NtHQT* overexpressing lines reached ∼46.2 ± 5.3 mg/g DW, which was ∼1.4 times higher than the *AtPAL2*, and 3.0 times the WT plants (data not shown). The average rutin content in the *AtPAL2/NtHQT* plants reached a level of 3.5 ± 0.5 mg/g DW, which was of no difference to the amount of rutin accumulated in the *AtPAL2* overexpression lines.
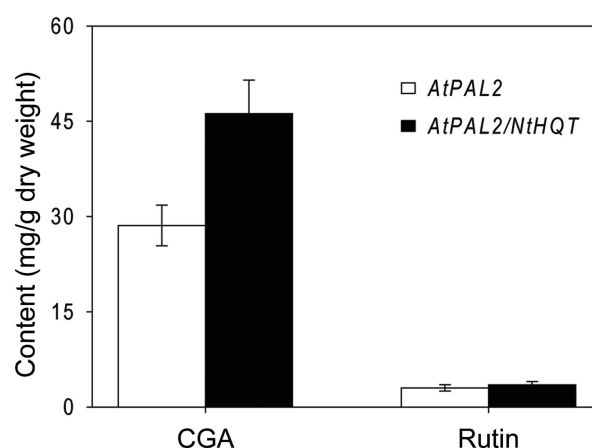

Fig. 4 Quantification of the two major polyphenols in *AtPAL2* and *AtPAL2/NtHQT* overexpression tobacco leaves   Leaf samples were taken at 45–50 days after sowing. Samples from 12 plants of each genotype were pooled and each value represents the mean ± SD.
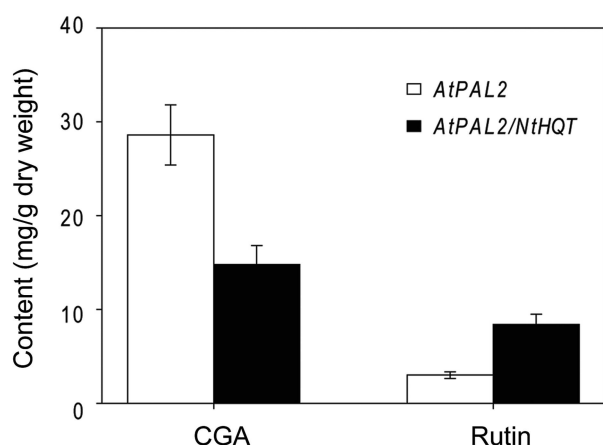
Fig. 5 Quantification of the two major polyphenols in *AtPAL2* and *AtPAL2/NtHQT* tobacco leaves   Leaf samples were taken at 45–50 days after sowing. Samples from 12 plants of each genotype were pooled and each value represents the mean ± SD.

Gene silencing of *NtHQT* in *AtPAL2* helped to increase rutin production

To determine the effects of silencing of *NtHQT* in the *AtPAL2* overexpression plants, *NtHQT* silencing plant (line HS2) was crossed with *AtPAL2* (line P3) (see the Materials and methods section), and the CGA and rutin contents in the leaves are shown in **Fig. 5**. The CGA content (14.8 ± 2.0 mg/g DW) in the *AtPAL2/NtHQT* line was ∼51% that of the *AtP4AL2/NtHQT* lines, whereas the rutin content reached 8.4 ± 1.1 mg/g DW, which was 2.8 times higher than that in the *AtPAL2* lines.

Discussion

Products of the phenylpropanoid pathway are structurally and functionally diverse and are synthesized in response to both developmental and environmental cues [25–27]. PAL catalyzes the first step of the phenylpropanoid pathway and is typically encoded by a small multigene family. In species such as *Oenothera*, only one of the two isoforms of PAL was involved in flavonoid biosynthesis [28], which suggested that in species possessing multiple PAL isoforms, the flux into various branches of phenylpropanoid pathway might be regulated by these isoforms either individually or coordinately. In addition, the fact that individual members of the *PAL* gene family are expressed differently during plant development and in response to different stress stimulus suggested that certain *PAL* genes may associate preferentially with specific multienzyme complexes to control the flux of metabolites through the different branches of the phenylpropanoid pathway [29]. Despite of all this investigations and suggestions, the metabolic significance of these is usually unknown. In Howles' report [18], overexpression of bean *PAL2* gene resulted in increased level of CGA but not rutin. We reported here the overexpression of *AtPAL2* gene resulted in the increase of both CGA and rutin in transgenic tobacco. The results that we got here gave the first time direct evidence of the idea that although functional redundancy might exist for the PAL family, different *PAL* gene (or different isoforms of *PAL* gene) might be involved into different branches of phenylpropanoid pathway.

Increasing evidence of healthy related functions of polyphenols in dietary has made metabolic engineering of phenylpropanoid pathway the subject of investigation in recent years [27]. A number of enzymes and the genes encoding these enzymes had been cloned and their roles had been investigated. So far, most of the researches concerned the alternation of flux distribution among different branches of this pathway. Since the total flux into this pathway is unchanged [30], the increase of flux into one branch of the pathway will normally lead to the decrease of flux into other branches within this pathway [31]. The advantage of overexpression of *PAL* is that it can increase the total flux into this pathway, so simultaneously increased accumulation of bioactives in different branches of this pathway could be achieved (**Fig. 3**). We also demonstrated that by combined overexpression of PAL with the overexpression/silencing of a specific branch of the pathway, further increase of the specific bioactive(s) can be obtained. These strategies can be used as for the metabolic engineering of multi-branch pathway(s) such as the phenylpropanoid pathway to produce functional food with increased polyphenols.

Funding

References

1 Haslam E. Practical Polyphenolics: From Structure to Molecular Recognition and Physiological Action. Cambridge: Cambridge University Press, 1998.

2 Harbone JB. The Flavonoids. Advances in Research since 1966, 1st ned. London: Chapman and Hall, 1986.

3 Sugihara N, Arakawa T, Ohnishi M and Furuno K. Anti- and pro-oxidative effects of flavonoids on metal-induced lipid hydroperoxide-dependent lipid peroxidation in cultured hepatocytes loaded with alpha-linolenic acid. Free Radic Biol Med 1999, 27: 1313–1323.

4 Dugas AJ, Castaneda-Acosta J, Bonin GC, Price KL, Fischer NH and Winston GW. Evaluation of the total peroxyl radical-scavenging capacity of flavonoids: structure-activity relationships. J Nat Prod 2000, 63: 327–331.

5 Duthie G and Crozier A. Plant-derived phenolic antioxidants. Curr Opin Lipidol 2000, 11: 43–47.

6 Ng TB, Liu F and Wang ZT. Antioxidative activity of natural products from plants. Life Sci 2000, 66: 709–723.

7 Hollman PC and Katan MB. Health effects and bioavailability of dietary flavonols. Free Radic Res 1999, 31: S75–S80.

8 Shih H, Pickwell GV and Quattrochi LC. Differential effects of flavonoid compounds on tumor promoter-induced activation of the human CYP1A2 enhancer. Arch Biochem Biophys 2000, 373: 287–294.

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

10 Trevisanato SI and Kim YI. Tea and health. Nutr Rev 2000, 58: 1–10.

11 Commenges D, Scotet V, Renaud S, Jacqmin-Gadda H, Barberger-Gateau P and Dartigues JF. Intake of flavonoids and risk of dementia. Eur J Epidemiol 2000, 16: 357–363.

12 Hertog MG, Feskens EJ and Kromhout D. Antioxidant flavonols and coronary heart disease risk. Lancet 1997, 349: 699.

13 Tamagnone L, Merida A, Stacey N, Plaskitt K, Parr A, Chang CF and Lynn D, et al. Inhibition of phenolic acid metabolism results in precocious cell death and altered cell morphology in leaves of transgenic tobacco plants. Plant Cell 1998, 10: 1801–1816.

14 Sawa T, Nakao M, Akaike T, Ono K and Maeda H. Alkylperoxyl radical-scavenging activity of various flavonoids and other phenolic compounds: implications for the anti-tumor-promoter effect of vegetables. J Agric Food Chem 1999, 47: 397–402.

15 McCarty MF. Nutraceutical resources for diabetes prevention-an update. Med Hypotheses 2005, 64: 151–158.

16 Lopez-Lopez G, Moreno L, Cogolludo A, Galisteo M, Ibarra M, Duarte J and Lodi F, et al. Nitric oxide (NO) scavenging and NO protecting effects of quercetin and their biological significance in vascular smooth muscle. Mol Pharmacol 2004, 65: 851–859.

17 Galisteo M, Garcia-Saura MF, Jimenez R, Villar IC, Zarzuelo A, Vargas F and Duarte J. Effects of chronic quercetin treatment on antioxidant defence system and oxidative status of deoxycorticosterone acetate-salt-hypertensive rats. Mol Cell Biochem 2004, 259: 91–99.

18 Howles PA, Sewalt V, Paiva NL, Elkind Y, Bate NJ, Lamb C and Dixon RA. Overexpression of L-phenylalanine ammonia-lyase in transgenic tobacco plants reveals control points for flux into phenylpropanoid biosynthesis. Plant Physiol 1996, 112: 1617–1624.

19 Niggeweg R, Michael AJ and Martin C. Engineering plants with increased levels of the antioxidant chlorogenic acid. Nat Biotechnol 2004, 22: 746–754.

20 Luo J, Nishiyama Y, Fuell C, Taguchi G, Elliott K, Hill L and Tanaka Y, et al. Convergent evolution in the BAHD family of acyl transferases: identification and characterization of anthocyanin acyl transferases from Arabidopsis thaliana. Plant J 2007, 50: 678–695.

21 D'Cunha GB, Nair PM and Satyanarayan V. Purification of phenylalanine ammonia lyase from Rhodotorula glutinis. Phytochem 1996, 42: 17–20.

22 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976, 72: 248–254.

23 Martin C, Carpenter R, Sommer H, Saedler H and Coen ES. Molecular analysis of instability in flower pigmentation of Antirrhinum majus, following isolation of the pallida locus by transposon tagging. EMBO J 1985, 4: 1625–1630.

24 van den Berg R, Haenen GRMM, van den Berg H and Bast A. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. Food Chem 1999, 66: 511–517.

25 Dixon RA and Paiva NL. Stress-induced phenylpropanoid metabolism. Plant Cell 1995, 7: 1085–1097.

26 Hahlbrock K and Scheel D. Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Physiol Plant Mol Biol 1989, 40: 347–369.

27 Raes J, Rohde A, Christensen JH, Van de Peer Y and Boerjan W. Genome-wide characterization of the lignification toolbox in Arabidopsis. Plant Physiol 2003, 133: 1051–1071.

28 Neumann G and Schwemmle B. Flavonoids from Oenothera seedlings—identification and extranuclear control of their biosynthesis. J Plant Physiol 1993, 142: 135–143.

29 Deshpande AS, Surendranathan KK and Nair PM. The phenylpropanoid pathway enzymes in Solanum tuberosum exist as a multienzyme complex. Indian J Biochem Biophys 1993, 30: 36–41.

30 Muir SR, Collins GJ, Robinson S, Hughes S, Bovy A, Ric De Vos CH and van Tunen AJ, et al. Overexpression of petunia chalcone isomerase in tomato results in fruit containing increased levels of flavonols. Nat Biotechnol 2001, 19: 470–474.

31 Joung JY, Kasthuri GM, Park JY, Kang WJ, Kim HS, Yoon BS and Joung H, et al. An overexpression of chalcone reductase of Pueraria montana var. lobata alters biosynthesis of anthocyanin and 5′-deoxyflavonoids in transgenic tobacco. Biochem Biophys Res Commun 2003, 303: 326–331.