

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 OD_{530} unite = 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[•]) in 6 min. The bluegreen ABTS[•] 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[•] 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 μ l ABTS[•] solution and A_{734} was measured. This was compared with a blank where 10 μ l of the solvent was added to 990 μ l of the ABTS[•] 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 μ g/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. tabacum* 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) via *Agrobacterium*-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

in lines P2 and P4. This might be 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 $\mu\text{mol Trolox/g DW}$ 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

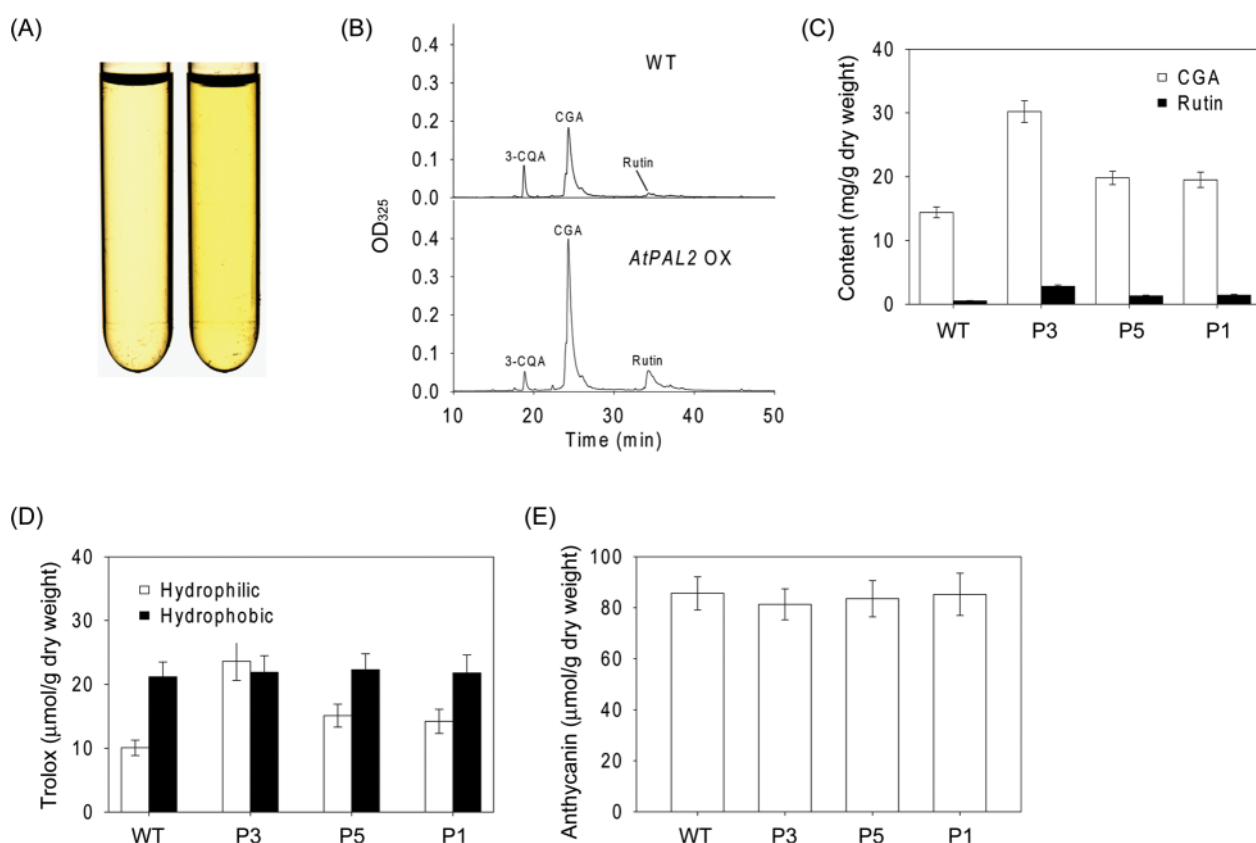


Fig. 3 Phenotype of *AtPAL2* overexpression tobacco lines (A) Pictures of extracts of the soluble polyphenols in the wild type (WT) (left) and *AtPAL2* (right) overexpression tobacco leaves. (B) HPLC analysis of soluble phenolics in leaves of WT and typical *AtPAL2* overexpression tobacco line. 3-CQA, 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 \pm 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 $\sim 46.2 \pm 5.3$ 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.

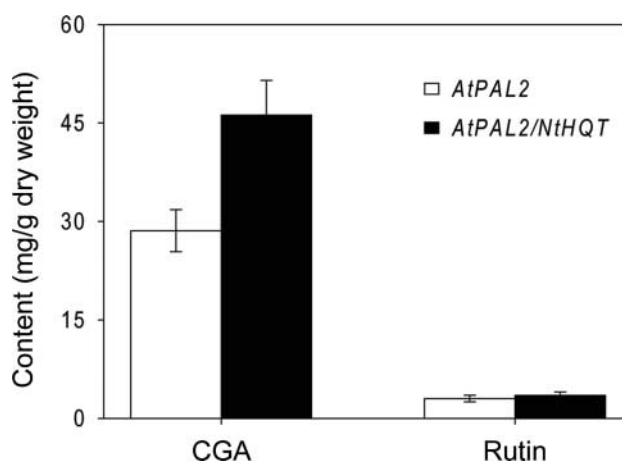


Fig. 4 Quantification of the two major polyphenols in *AtPAL2* and *AtPAL2/NtHQT* overexpression tobacco leaves Leaf samples were taken at 45–50 days after sowing. Samples from 12 plants of each genotype were pooled and each value represents the mean \pm SD.

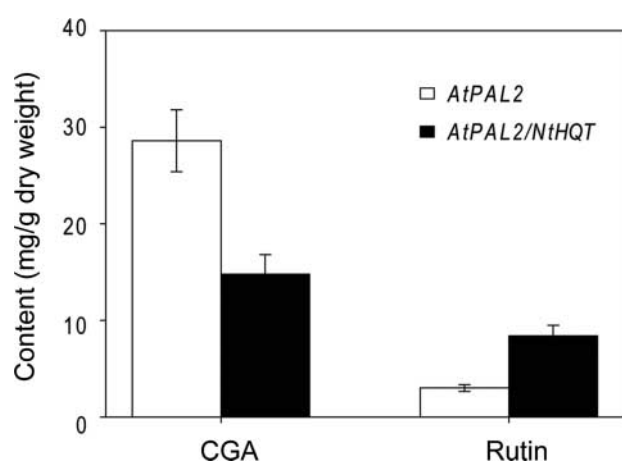


Fig. 5 Quantification of the two major polyphenols in *AtPAL2* and *AtPAL2/NtHQT* tobacco leaves Leaf samples were taken at 45–50 days after sowing. Samples from 12 plants of each genotype were pooled and each value represents the mean \pm SD.

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 $\sim 51\%$ that of the *AtPAL2* 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.

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