

Polyphenols Displaying Tyrosinase Inhibition from the Seed of *Psoralea corylifolia*

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Received April 7, 2010; Accepted April 30, 2010

Three chalcones and two flavanones displaying tyrosinase inhibitory activity were isolated from the seed of *Psoralea corylifolia*, a cultivated edible plant. The isolated compounds were elucidated as known isovabachalcone, 4'-*O*-methylbavachalcone, isobavachromene, corylifolin, and bavachinin. The inhibitory potencies of the polyphenols toward monophenolase activity of mushroom tyrosinase were investigated. The inhibitory concentration (IC₅₀) values of compounds 1-5 for monophenolase activity were determined to be 12.3, 48.8, 15.8, 23.6, and 143.9 μM, respectively. The potent inhibitors 1-4 exhibited the competitive inhibition characteristics in analysis of Lineweaver-Burks and Dixon-plot.

Key words: monophenolase, mushroom tyrosinase, prenylated chalcone, *Psoralea corylifolia*

Tyrosinase (1.14.18.1), widely distributed in nature, is a metalloenzyme oxidase which catalyzed two distinct reactions of melanin synthesis; the hydroxylation of monophenol and the oxidation of *o*-diphenol to the corresponding *o*-quinon [Prota, 1988]. The ubiquitous enzyme initiates the synthesis of melanin and is responsible for the browning that occurs upon bruising or long-term storage of vegetables, fruits, and mushrooms. Furthermore, tyrosinase is not only responsible for browning of hair and skin pigmentation [Del Marmol *et al.*, 1996; Sturm *et al.*, 2001], but also it may play a role in cancer and neurodegenerative diseases in mammals [Cavalieri *et al.*, 2002]. As, tyrosinase inhibitors have recently attracted a lot of interest due to decrease of the hyperpigmentation resulting from the enzyme action. Hence, tyrosinase inhibitors are supposed to have broad applications in medicinal and cosmetic products.

Many efforts have been spent in the search for feasible and effective tyrosinase inhibitors. There is a concerted effort to search for naturally occurring tyrosinase inhibitors from plants, because plants constitute a rich source of

bioactive chemicals and many of them are largely free from harmful adverse effects. It is well known that tyrosinase can be inhibited by aromatic aldehydes [Jiménez *et al.*, 2001] and acids [Robit *et al.*, 1997; Huang *et al.*, 2006], tropolone [Valero *et al.*, 1991] and kojic acid [Canaens *et al.*, 1994]. Our previous work have found effective inhibitors from natural sources: lavandululated flavanones from *Sophora flavescens* [Ryu *et al.*, 2008], prenylated flavones from *Morus lhou* [Jeong *et al.*, 2009], prenylated xanthenes from *Cudrania tricuspidata* [Oh *et al.*, 2007] and 1,3-diphenylpropanoids from *Broussonetia kazinoki* [Baek *et al.*, 2009].

In our continuing search for tyrosinase inhibitors from medicinal plants, we found that the chloroform extracts from the seed of *Psoralea corylifolia* (*P. corylifolia* Legume family) can remarkably inhibit tyrosinase activity (63% at 250 μg/mL). *P. corylifolia* is plentiful sources of polyphenol-rich plant, which have been reported antibacterial [Yin *et al.*, 2004], antifungal [Ragendra *et al.*, 2004] and antioxidative [Jiangning *et al.*, 2005] activities. We recently reported that this plant derived polyphenols exhibit α-glucosidase inhibition [Oh *et al.*, 2010].

In this study, we isolated five known polyphenols from the chloroform extract of the seed of *P. corylifolia*, and identified their structures using spectroscopic methods (Fig. 1). All isolated compounds were additionally evaluated

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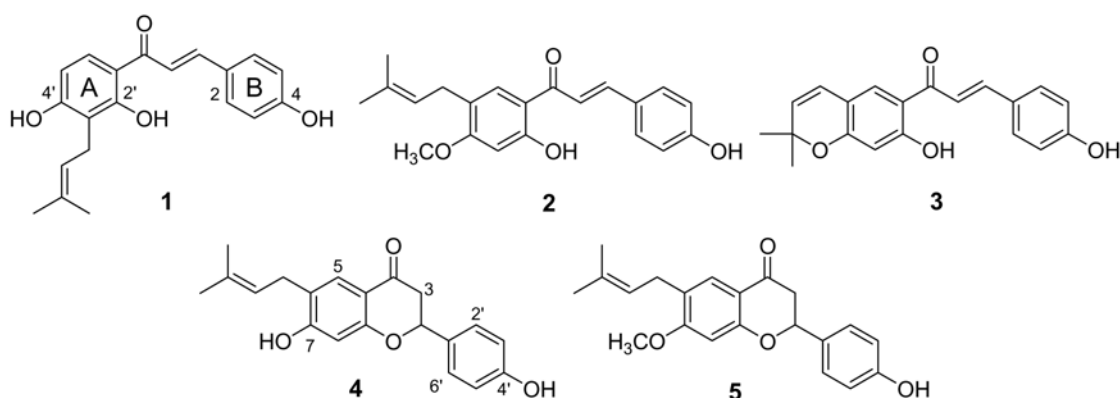


Fig. 1. Chemical structure of the *P. corylifolia* isovabachalcone (1), 4'-*O*-methylbavachalcone (2), isobavachromene (3), corylifolin (4), and bavachinin (5).

for their inhibitory activities and kinetics mode on tyrosinase inhibition.

Materials and Methods

Chemicals and materials. Organic solvents used for isolation were of first grade and the stock solution and buffers were prepared with Milli Q water. Methanol, acetonitrile and acetic acid for HPLC were purchased as of analytical grade from J.T. Baker (Phillipsburg, NJ, USA). Column chromatography was carried out using silica gel (230-400 mesh, Merck), RP-18 (ODS-A, 12 nm, S-150 mM, YMC), and Sephadex LH-20 (Amersham Biosciences). For biochemical assays, mushroom tyrosinase (EC 1.14.18.1), L-tyrosine were purchased from Sigma Chemical Co. (St. Louis, MO). *P. corylifolia* [imported from Myanmar, as permitted by Korea Food and Drug Administration (KFDA)] (approximately 2.8 kg) was purchased from a market.

Instruments. UV spectra were measured on a Beckman DU650 spectrophotometer (Beckman Coulter, Fullerton, CA). ^1H and ^{13}C NMR, as well as 2D NMR data, were obtained on a Bruker AM 500 (^1H NMR at 500 MHz, ^{13}C NMR at 125 MHz) spectrometer (Bruker, Karlsruhe, Germany) in CDCl_3 , and acetone- d_6 with TMS as internal standard. EIMS, HREIMS were obtained on a JEOL JMS-700 mass spectrometer (JEOL, Tokyo, Japan). Qualitative analyses were made using a Perkin-Elmer HPLC S200 (Perkin-Elmer, Bridgeport, CT). LC/MS was measured in a 3200 Q Trap LC/MS/MS System (Applied Biosystems, Lincoln, CA). Reagent grade chemicals were purchased from Sigma Chemical Co. (St. Louis, USA).

Tyrosinase inhibitory activity. Mushroom tyrosinase (EC 1.14.18.1) (Sigma Chemical Co., St. Louis, MO) was used as described [Jeong *et al.*, 2009] previously with some modifications, using either, L-tyrosine (monophenolase) as substrate. In spectrophotometric experiments, enzyme

activity was initial velocity (v_i) monitored by observing dopachrome formation at 475 nm with a UV-vis spectrophotometer (Spectro UV-vis double beam; UVD-3500, Labomed, Inc.) at 30°C. All samples were first dissolved in EtOH at 10 mM. First, 66 μL of a 2.7 mM L-tyrosine ($K_m=180 \mu\text{M}$) aqueous solution was mixed with 824 μL of 0.25 M phosphate buffer (pH 6.8). Then, 100 μL of the sample solution and 10 μL of the same phosphate buffer solution containing mushroom tyrosinase (144 units) were added in this order to the mixture. Each assay was conducted as three separate replicates. The inhibitor concentration leading to 50% activity loss (IC_{50}) was obtained by fitting experimental data to the logistic curve by eq 1 [Copeland, 2000]:

$$\text{Activity (\%)}=100[1/(1+([I] \text{IC}_{50}))] \quad (1)$$

Statistical analysis. All experiments were repeated at least three times. Results are expressed as mean \pm SD (standard deviation). The statistical significance of differences between groups was evaluated for two parallel experiments using the student's *t*-test.

HPLC analysis. Quantification of the relative abundance of the compounds assayed in this manuscript within the crude seed extract was carried out by HPLC (Perkin-Elmer 200 series, Perkin-Elmer Co., Bridgeport, CT) using a SPHERI-5 RP-18 column (4.6 \times 250 mm, 5 μm , Perkin-Elmer, USA). Absorbances were measured at 310 nm. About 20 mL of 100 $\mu\text{g}/\text{mL}$ of crude seed extract were loaded onto the column. The initial mobile phase consisted of 20% solvent B (acetonitrile) in solvent A [0.1% acetic acid in water (A)]. This was linearly increased to 60% B over a period of 1 h at a constant flow rate of 1 mL/min. The column was then held at 60% B for 10 min.

Extraction and isolation. The air-dried root bark (2.8 kg) of *P. corylifolia* were chopped and extracted with chloroform (5 L \times 4) at room temperature for 7 day. The

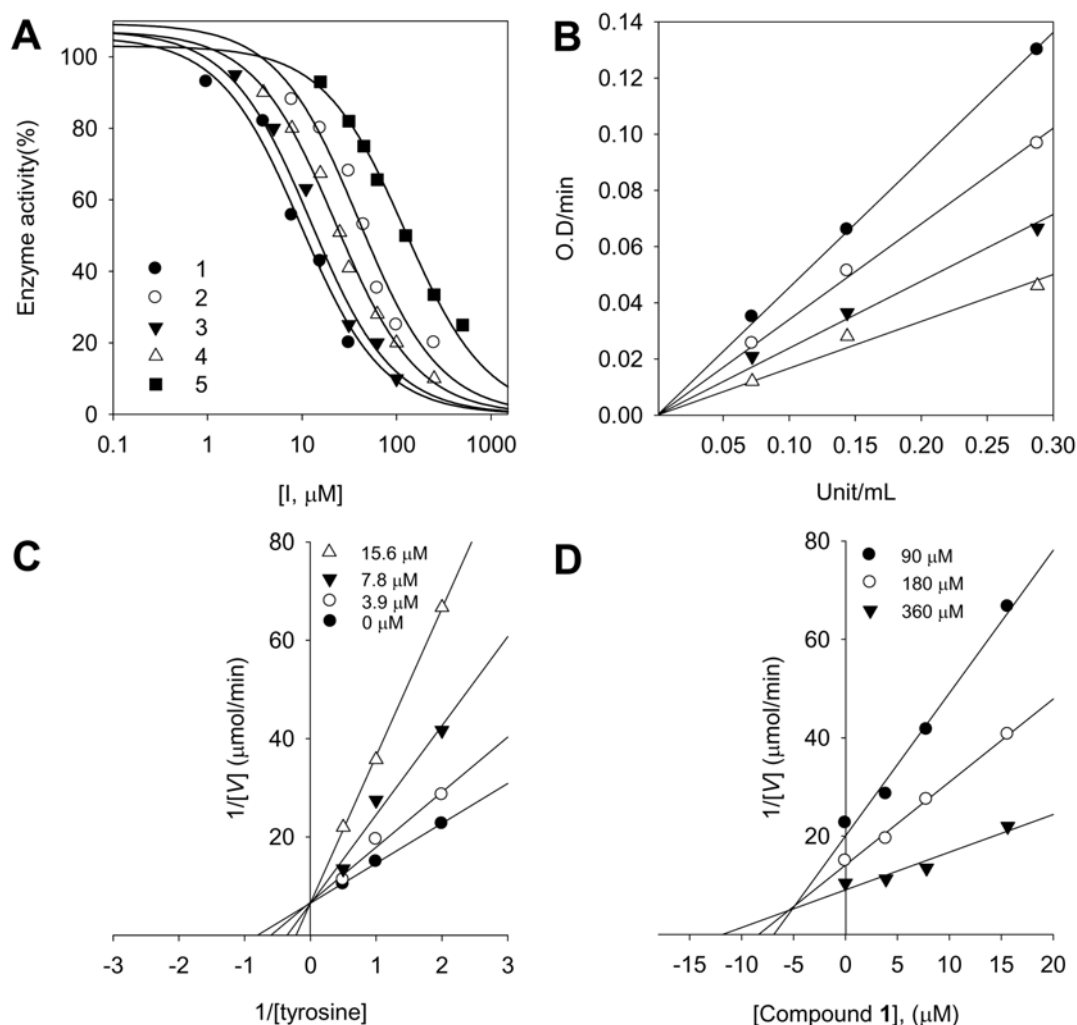


Fig. 2. (A) Effect of compounds (1, 2, 3, 4, and 5) on the activity of tyrosinase for the catalysis of L-tyrosine. (B) Relationship between the catalytic activity of tyrosinase and concentrations of compound 1. Concentrations of compound 1 for lines from top to bottom were 0, 3.9, 7.8 and 15.6 μM , respectively. (C) Lineweaver-Burk plots for the inhibition of compounds 1 on the monophenolase activity of tyrosinase. Conditions were as follows: 180 μM L-tyrosine, 144 units of tyrosinase, 0.25 M phosphate buffer (pH 6.8), at 30°C. In the presence of different concentrations of compounds for lines from bottom to top: for compound 1, 0, 3.9, 7.8 and 15.6 μM . (D) Dixon plots for the inhibition of compound 1 on the monophenolase activity of tyrosinase. In the presence of different concentrations of substrate for lines from bottom to top: 360, 180, and 90 μM .

combined filtrate was concentrated in vacuo to yield a dark brown gum (386.4 g). This crude extract (30.4 g) was fractionated by silica gel flash CC employing a gradient of hexane to EtOAc, resulting in 11 fractions (fractions A-K). The pure compounds 1-5 have been isolated by different chromatographic methods and characterized as described previously [Oh *et al.*, 2010].

Compound 1. ^{13}C NMR (125 MHz) δ 18.3 (C-4''), 22.7 (C-1''), 26.3 (C-5''), 108.4 (C-3'), 114.9 (C-1'), 116.5 (C-5'), 117.1 (C-3 and 5), 119.0 (C- α), 123.7 (C-2''), 128.1 (C-1), 130.7 (C-2'), 131.9 (C-3''), 132.1 (C-2 and 6), 145.3 (C- β), 161.3 (C-4), 163.3 (C-4'), 165.1 (C-6'), 193.4 (C=O).

Compound 2. ^{13}C NMR (125 MHz) δ 18.3 (C-4''),

26.3 (C-5''), 29.8 (C-1''), 56.7 (4-OCH₃), 100.4 (C-5'), 114.6 (C-1'), 117.2 (C-3 and 5), 118.8 (C- α), 122.8 (C-3'), 124.2 (C-2''), 128.0 (C-1), 131.6 (C-2'), 132.1 (C-2 and 6), 132.9 (C-3''), 145.5 (C- β), 161.4 (C-4), 165.4 (C-4'), 166.4 (C-6'), 193.3 (C=O).

Compound 3. ^{13}C NMR (125 MHz) δ 29.0 (C-7'' and 8''), 79.2 (C-6''), 109.7 (C-3'), 110.7 (C-5'), 115.7 (C-1'), 117.1 (C-4'), 117.4 (C-3 and C-5), 118.5 (C- α), 128.1 (C-1), 129.8 (C-5'), 132.4 (C-2 and 6), 132.8 (C-2'), 146.5 (C- β), 161.3 (C-4'), 162.1 (C-6'), 162.3 (C-6), 194.3 (C=O).

Compound 4. ^{13}C NMR (125 MHz) δ 18.2 (C-4''), 26.3 (C-5''), 29.7 (C-1''), 45.2 (C-3), 80.9 (C-2), 103.6 (C-2), 115.3 (C-4a), 116.5 (C-3'), 116.5 (C-5'), 123.6 (C-2''),

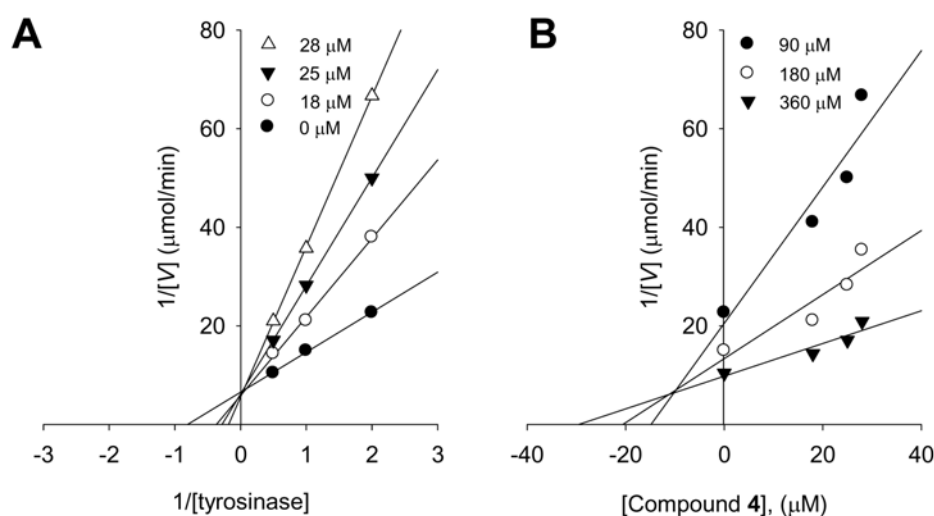


Fig. 3. Lineweaver-Burk and Dixon plots for the inhibition of compound 4 on the monophenolase activity of tyrosinase.

124.2 (C-3''), 128.6 (C-5), 129.3 (C-2'), 129.3 (C-6'), 131.9 (C-1'), 133.4 (C-7), 158.9 (C-4'), 163.2 (C-8a), 163.1 (C-6), 191.0 (C-4).

Compound 5. ^{13}C NMR (125 MHz) δ 18.2 (C-4''), 26.3 (C-5''), 28.2 (C-1''), 44.5 (C-3), 56.2 (7-OCH₃), 80.2 (C-2), 99.2 (C-8), 114.3 (C-4a), 116.1 (C-3', C-5'), 122.1 (C-2''), 125.4 (C-6), 127.5 (C-5), 128.4 (C-2', 6'), 131.2 (C-1'), 133.5 (C-3'), 156.7 (C-4'), 162.9 (C-8a), 164.7 (C-7), 192.0 (C=O).

Results and Discussion

Activity-guided fractionation of the chloroform extract gave five phenolic compounds (**1-5**) which were purified over silica gel and octadecyl-functionalized silica gel as delineated above. Through analysis of spectroscopic data, including 2D NMR spectra and HREIMS, the compounds were identified as isovabachalcone (**1**), 4'-*O*-methylbavachalcone (**2**), isobavachromene (**3**), corylifolin (**4**), and bavachinin (**5**) (Fig. 1). The discussion of structural identification will focus on compound **1** which emerged to be the most potent tyrosinase inhibitor. Compound **1** has the molecular formula C₂₀H₂₀O₄ and eleven degrees of unsaturation, as deduced from HREIMS m/z 324.1365 [M⁺]. The ^1H and ^{13}C NMR data in conjunction with a DEPT experiments delineated the presence of 20 carbon atoms, consisting of the following functional groups: one carbonyl, one methylene (sp³), nine methines (sp²), two methyls, and 7 quaternary carbons. The ^{13}C NMR data enable carbons corresponding to the carbonyl and eight C-C double bonds to be identified, and thus accounted for nine of the eleven degrees of unsaturation. The extra two degrees of unsaturation were ascribed to a two cyclic system. The chalcone skeleton was deduced from α,β -

unsaturated C-C double in dH 7.68 (1H, d, $J=15.0$ Hz) and dH 7.78 (1H, d, $J=15.3$ Hz). The presence of isoprenyl group was deduced from successive connectivities from H-1'' (dH 3.33) to H-5'' (dH 1.72) in the ^1H - ^1H COSY spectrum. HMBC correlation H-1'' (dH 3.33) and C-3' (dH 108.4) proved the location of the isoprenyl group (Fig. 4). Thus, compound **1** was identified as 2',4',4'-trihydroxy-3'-(3,3-dimethylallyl)-chalcone, as called isovabachalcone.

In the preliminary screening, we observed that chloroform extracts of the seeds of *P. corylifolia* showed significant inhibition of L-tyrosinase oxidation. More detailed bioassays of the isolated compounds were subsequently conducted. All compounds (**1-5**) showed a dose-dependent inhibitory effect on monophenolase activity. As the concentrations of the inhibitors were increased, the residual enzyme activity drastically diminished (Fig. 2A). As shown in Table 1, IC₅₀ values of 12.3, 48.8, 15.8, 23.6, and 143.9 μM , were obtained. The potency of compound **1** (IC₅₀=12.3 μM) can be favorably compared with commercially available inhibitor currently used as cosmetics, such as kojic acid (IC₅₀=16.2 μM).

The inhibition of mushroom tyrosinase by compound **1** is illustrated in Fig. 2B, representatively. Plots of residual enzyme activity versus enzyme concentration at different concentration of compound **1** gave a family of straight lines with a y -axis intercept of 0 and increasing concentration of compound **1** resulted in a reduction of the slopes of the line. This result proved that compound **1** is reversible inhibitor. We progressed to analyze the mode of inhibition using Lineweaver-Burk and Dixon plots (Fig. 2C and D), which revealed that compound **1**, exhibited competitive inhibition because increasing concentration of substrate resulted in a family of line which intersected at a zero

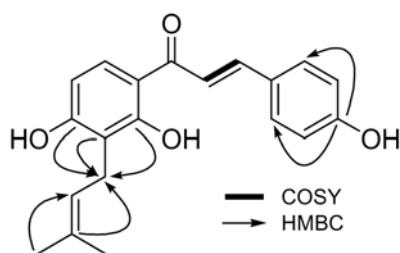


Fig. 4. COSY and key HMBC correlations of **1**.

Table 1. Inhibitory effects of compounds (**1-5**) on monophenolase activity of mushroom tyrosinase

Compound	Tyrosinase	
	IC ₅₀ ^a (μM)	Type of Inhibition K _i (μM) ^b
1	12.3±0.3	competitive (5.1±0.3)
2	48.8±0.5	competitive (13.3±1.2)
3	15.8±1.0	competitive (6.2±0.8)
4	23.6±0.1	competitive (9.8±0.2)
5	143.9±2.0	NT
Kojic acid	16.3	NT

^aAll compounds were examined in a set of experiments repeated three times; IC₅₀ values of compounds represent the concentration that caused 50% enzyme activity loss.

^bValues of inhibition constant.

^cNT, not tested

point on the *x*-axis. Other three potent inhibitors (**2-4**) also showed the competitive inhibition modes.

To set the importance of these inhibitors in a practical context, we performed a comparative analysis of the levels of individual tyrosinase inhibitory phenolic compounds within the native seed using HPLC analysis. The nature of each peak in the HPLC trace was doubly verified by comparison with retention time of the pure compound and also by LC/MS of each fraction. All compounds examined in this study were detected in the HPLC chromatogram, as shown in Fig. 5. Importantly, LC/MS analysis of each fraction shows characteristics consistent with our assigned structure. Moreover, the most active tyrosinase inhibitor **1**, and **5** were present in very high concentrations because they appeared as principal peaks in chromatogram, such as at 43.2 min, isobavaisoflavone (**1**), and 43.4 min, bavachinin (**5**).

In conclusion, five polyphenols displaying tyrosinase inhibitory activity were successfully isolated and purified from the seeds of *P. corylifolia*. The inhibitory potencies and capacities of these polyphenols toward monophenolase activity of mushroom tyrosinase were studied detail to unveil that they are all competitive inhibitors.

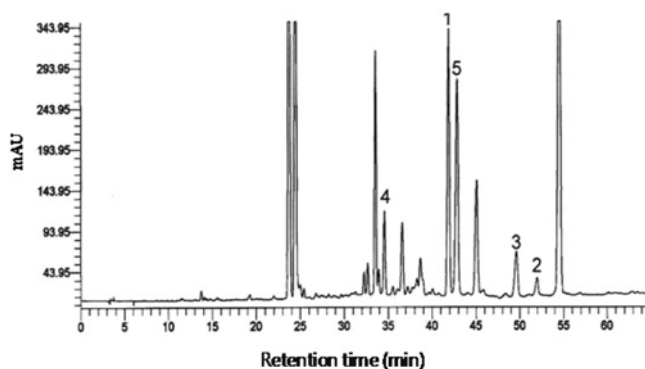


Fig. 5. Chromatogram of chloroform extract.

Acknowledgments. This work was supported by the National Research Foundation of Korea (NRF No. 20090081751) and financially supported by a grant (20080401-034-063-008-03) from BioGreen 21 project Programme, Republic of Korea. J.Y. Kim was supported by a scholarship from the BK21 program.

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