Table 2. Variation in DNA Sequences in ITS1, ITS2, and *trn*L/F of *Ephedra* Plants Collected in Xinjiang

Species	Genotype	Voucher no.	ITS1						ITS2	*trn*L/F	
			179	773	884	910	1004	1119	205	96	398
E. intermedia	Ei-I	23-20	T	T	A	A	C	A	G	C	A
	Ei-II	15-10, 17-10	T	C	A	C	C	G	G	C	A
	Ei-III	11-40, 12-30	T	C	A	C	T	R(A,G)	G	C	A
E. regeliana	Er	11-50, 12-20, 13-10	T	C	A	C	C	R(A,G)	G	C	C
E. przewalskii	Ep-I	303	C	C	C	C	C	G	A	C	A
	Ep-II	11-10	C	C	C	C	C	G	R(A,G)	C	A
	Ep-III	306	C	C	C	C	C	G	G	C	A
	Ep-IV	11-31, 19-10	T	C	C	C	C	G	G	C	A
	Ep-V	13-40, 15-20	T	C	C	C	C	G	G	C	C
	Ep-VI	20-10, 21-10, 22-10	T	C	C	C	C	G	G	T	A

Nucleotide numbers were counted from the beginning of the respective regions.

Table 3. Content of Four Ephedrine Alkaloids, Norephedrine (NE), Pseudoephedrine (PE), Ephedrine (E), and Methylephedrine (ME), in Aerial Parts of *Ephedra* Plants Collected in Xinjiang

Species		Plant no.	Content (% of dry weight)				
			NE	PE	E	ME	NP+EP+P
E. intermedia	Ei-I	23-20	0.009	1.56	ND	ND	1.57
	Ei-II	15-10	0.048	0.71	0.33	ND	1.09
		17-10	0.031	1.54	0.24	ND	1.81
	Ei-III	11-40	0.011	1.42	0.031	ND	1.46
		12-30	0.088	0.15	0.22	ND	0.46
E. przewalskii		All specimens	ND	ND	ND	ND	
E. regeliana		All specimens	ND	ND	ND	ND	

ND, not detected.

Nucleotide Variation in ITS1, ITS2, and *trn*L/F The results of DNA analysis of ITS1, ITS2, and *trn*L/F are shown in Table 2. The nucleotide numbers of respective regions were found to be 1139 bp for ITS1, 246 bp for ITS2, and 465 bp for *trn*L/F.

In *E. intermedia*, we found three genomic variations, which were correlated with morphologic change. One specimen, 23-20 (genotype Ei-I), which was collected in the eastern part, showed an identical sequence as in the previous report. The other four specimens with two morphologic types had substitutions at positions 773 and 910 in ITS1 compared with 23-20. Specimens 11-40 and 12-30 (genotype Ei-III) had an additional substitution at position 1004 and an ambiguity of adenine/guanine at 1119, where specimens 15-10 and 17-10 (genotype Ei-II) had guanine.

In contrast to three specimens of *E. przewalskii* collected in Gansu and Qinghai, which had identical sequences, six genotypes were found in Xinjiang. Specimen 303 (genotype Ep-I) showed the same sequences as that of *E. przewalskii* in Gansu and Qinghai. Ep-I, -II, and -III, which had the same sequences in ITS1 and *trn*L/F, showed variation in ITS2. Specimen 11-10 showed almost equal intensity of adenine and guanine signals at position 205 in ITS2. The other specimens of *E. przewalskii* had substitutions at position 205 from adenine of genotype Ep-I to guanine. Ep-IV, Ep-V and Ep-VI were identical in ITS1 and -2, but not in *trn*L/F. The specimens of genotype Ep-V had identical *trn*L/F sequences to those of *E. regeliana*. The specimens of genotype Ep-VI collected in the south of the Taklimakan Desert had substitutions from the cytosine of genotype Ep-I to thymine at position 96 of *trn*L/F.

Three specimens of *E. regeliana* had the same nucleotide sequences, including an ambiguity of adenine/guanine at position 1119 in ITS1.

Ephedrine Alkaloid Content The specimens of *E. intermedia* contained ephedrine alkaloids (Table 3), although variation in content was observed. The total content of ephedrine alkaloids varied from 0.46% to 1.81%. On the other hand, although it seems that huge gaps between the habitats resulted in intraspecific variation in DNA sequences, no specimen of *E. przewalskii* contained detectable amounts of ephedrine alkaloids, and neither did the *E. regeliana* specimens.

DISCUSSION

Desert covers the most of Xinjiang. It is divided into two parts by Tianshan Mountains, the Gureban-tonggute Desert and Jungger Basin in the north, and the Taklimakan Desert and Tarim Basin in the south. Vegetation in the peripheral areas of the desert consists of thin scrub and dwarf woodlands dominated by representative desert plants such as saxoul (*Haloxylon ammodendron*) and *E. przewalskii*.

Many studies reported that there was intraspecific variation in *trn*L/F according to geographic change.[5—7] In our survey, we found *E. przewalskii* had intraspecific variation in

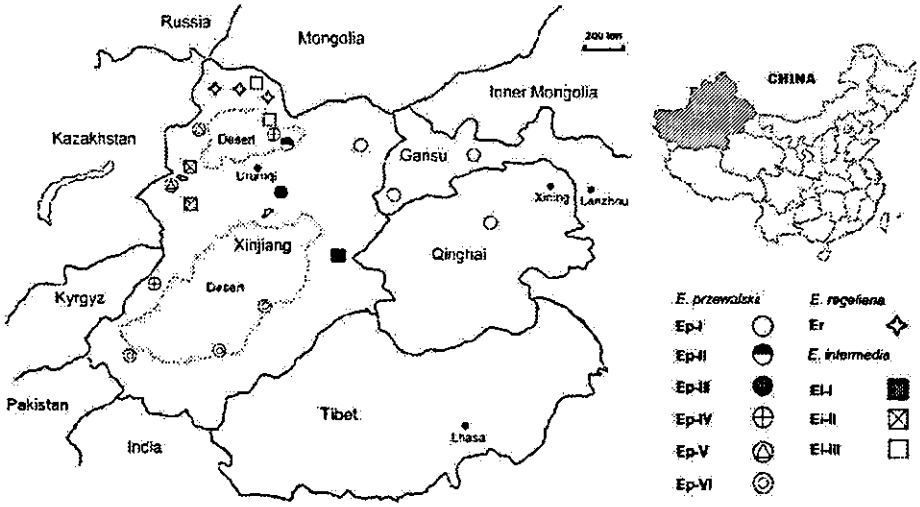

Fig. 2. Collection Sites of *Ephedra* Plants in Xinjiang

ITS1, ITS2, and *trnL/F* and there was a close relationship between genomic variation and habitat location. Only specimen 303, which was collected near the border of Gansu and Xinjiang, has the same sequences as specimens collected in Gansu and Qinghai. The habitats of specimens belonging to the same genotype were near each other except for that of genotype Ep-IV. We found specimens with genotype Ep-I, Ep-II, Ep-III, Ep-IV, and Ep-V in the north, and genotype Ep-IV and Ep-VI in the south part. Proceeding along northern or southern edge of the deserts from east to west, DNA sequences of the specimens of *E. przewalskii* continuously changed. Geographic factors may thus affect DNA sequences.

E. regeliana grows in the northern part of Xinjiang. *E. regeliana* had DNA sequences similar to *E. przewalskii* genotype Ep-V, whose habitat is also located in the northern part. Interestingly, there were no detectable amounts of ephedrine alkaloids in *E. regeliana*, although there was a report that *E. regeliana* collected from Pakistan contained ephedrine and pseudoephedrine.[8] There may be a close relationship between *E. regeliana* and *E. przewalskii*, although this conclusion can not be deduced from the morphology.

Three genotypes of *E. intermedia* were different morphologically as well as genetically. In spite of morphologic and genetic variations, they all showed high contents of ephedrine alkaloids. Furthermore, except for specimen 12-30, they contained more pseudoephedrine than ephedrine, which is characteristic of *E. intermedia*.[9]

From the survey of *Ephedra* plants in Xinjiang, we conclude that medically useful *Ephedra* plants are still abundant, and that they show variations compared with those from the other eastern regions in China.

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白芷の調製法と化学的品質評価（第5報）保存中におけるフロクマリンの減少

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Preparation and Chemical Evaluation of Angelica dahurica Root (Part V) Decrement of Furanocoumarins during Preservation

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Angelica dahurica roots were preserved under various conditions for one year and were chemically evaluated by the contents of furanocoumarins. Eight linear furanocoumarins, i.e., psoralen, xanthotoxin, bergapten, byak-angelicol, oxypeucedanin, imperatorin, phellopterin and isoimperatorin, gradually decreased in the course of time during preservation except under dark and cold conditions. The contents of eight furanocoumarins decreased by UV irradiation for one week. The remarkable decrement of byak-angelicol and oxypeucedanin was observed when the powdered roots were heated at 50-60℃ for one week. The other six furanocoumarins were stable to heating. These findings showed that the decrement of eight furanocoumarins during preservation could be due to not only pyrolysis but also being bathed in light especially UV.

Keywords: Angelica dahurica root; furanocoumarin; preservation; decrement; UV

白芷はヨロイグサ *Angelica dahurica* BENTHAM et HOOKER（セリ科）の根を乾燥した生薬で，鎮痛薬とみなされる漢方処方及びその他の処方に少数例配合されている．白芷の成分として種々のクマリン誘導体が知られている[1]．

第2報[2]では入手年の明らかな白芷中のリニア型フロクマリン8種（Fig. 1に示すpsoralen, xanthotoxin, bergapten, byak-angelicol, oxypeucedanin, imperatorin, phellopterin及びisoimperatorin）をHPLCを用いて定量し，化学的に品質評価した．その結果，長期間室温に保存した試料は主成分であるbyak-angelicol及びoxypeucedanin含量が極めて低い傾向にあり，これらは保存中に分解すると考えられた．このことから，白芷中のフロクマリンは試料の保存条件及び保存期間の違いにより，その含量が大きく変動する可能性が示唆された[2]．

そこで今回は，北海道で育成したヨロイグサ[3]の乾燥根（白芷）を種々の条件で1年間保存し，その保存条件がフロクマリンの含量に及ぼす影響を経時的に調べた．さらに，フロクマリンの紫外線及び熱に対する安定性について検討を加えた．

実 験 方 法

1. 保存法別試料

北海道薬用植物栽培試験場圃場で育成した2年生ヨロイグサを2000年10月27日に掘上げた．水洗後，茎葉を除去し，透明アクリル板を張った無加温ハウス内で自然乾燥（11月2日～2001年1月9日）した後，温風乾燥（50℃，48時間）した．北海道立衛生研究所において超遠心粉砕機（Retsch社，ZM1，メッシュスクリーン穴径0.5 mm使用）で粉砕後，粉末を以下のA～Fの6条件で1年間保存した（各々5 g使用，2001年6月1日～2002年6月3日）．

A: ガラス瓶に入れ，冷蔵室内の暗所に保存した．

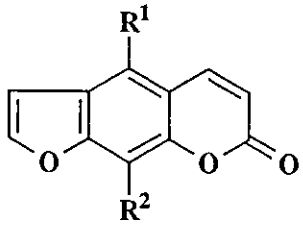

Fig. 1. Chemical Structure of Furanocoumarins.
Psoralen, $R^1=R^2=H$; Xanthotoxin, $R^1=H$ $R^2=OCH_3$; Bergapten, $R^1=OCH_3$ $R^2=H$; Byak-angelicol, $R^1=OCH_3$ R^2=3-methyl-2,3-epoxybutyloxy; Oxypeucedanin, R^1=3-methyl-2,3-epoxybutyloxy $R^2=H$; Imperatorin, $R^1=H$ R^2=3,3-dimethylallyloxy; Phellopterin, $R^1=OCH_3$ R^2=3,3-dimethylallyloxy; Isoimperatorin, R^1=3,3-dimethylallyloxy $R^2=H$.

B: ガラス瓶に入れ，実験室内［直射日光の当たらない本箱上，天井の蛍光灯（32 W×2，1年間の点灯時間は約2,700時間）から30 cm下］に放置した．

C: ポリ袋に二重にして入れ，Bのガラス瓶と並べて放置した．

D: ガラス瓶に入れ，ネット袋に入れた後，温室のガラス屋根から約40 cmの所に吊り下げた．

E: ポリ袋に二重にして入れ，Dのガラス瓶と共に温室内に放置した．

F: ガラス瓶に入れ，アルミ箔で二重に包んでから，Dのガラス瓶と共に温室内に放置した．

各々について7月2日，8月3日，9月3日，10月3日，11月2日，2002年2月4日及び6月3日に粉末の一部を取り出し，分析用試料とした．保存期間中の各試料を放置した場所の気温は，冷蔵室内で4.5℃，実験室内で15～32℃，温室内で6～58℃であった．

なお，ガラス瓶は400～360 nmの透過率が92%，360～280 nmでは波長が短くなるに従い漸次透過率が低下（310 nmで50%）し，280 nm以下の波長域の紫外線を透過しないネジ栓付遠心沈澱管（岩城硝子(株)，PYREX，50 ml，TE-32)[4]，ポリ袋はチャック付（伊藤忠サンプラス(株)，サンジップE-4，低密度ポリエチレン）を用いた．ポリ袋は気体透過性，透湿性を有し[5]，二重にして紫外線を照射（次項参照）したところ，その透過率は約80%であった．

2. 紫外線照射

上記粉末を以下のG～H′の4条件で紫外線照射あるいは暗所に放置した．照射は15 Wの殺菌灯（三共電気(株)，殺菌ランプGL15）から約20 cm離し（9 J/m²・sec），段ボール箱中で1週間行った（箱内温度24～28℃）．

G: シャーレ（直径12 cm）2枚に薬包紙を敷き，その各々の上に粉末約300 mgを薄く広げ，ふたをせずに直接照射した．

G′: シャーレ上の粉末約300 mgを，1週間放置した（箱内温度25～26℃）．

H: ガラス瓶[4]に粉末約650 mgを入れ，栓をした後，Gと共に照射した．

H′: ガラス瓶中の粉末約700 mgを，G′と共に1週間放置した．

3. 加熱処理

上記粉末を以下のI～Kの3条件で加熱処理した．加熱はヒーティングブロック（ヤマト科学(株)，HL-21型のアルミブロックを外し，温度計を差した段ボールで蓋をした）中で1週間行った．

I: ガラス瓶に粉末約650 mgを入れ，栓をし，アルミ箔で包んでから40±0.5℃に加熱した．

J: Iと同様の試料を50±0.5℃に加熱した．

K: Iと同様の試料を60±0.5℃に加熱した．

4. HPLCによるフロクマリンの定量

条件A～Fについては，各々約250 mgを分析用試料A～Fとした．各試料にメタノール10.0 mlを加え，超音波処理（ヤマト科学(株)，Branson B-32H，270 W，約50℃，20分間）し，遠心分離（3,000 rpm，10分間）後，上清を試験溶液とした[2]．

条件G～Kについては，各々約300 mgを分析用試料G～Kとした．各試料にメタノール/水混液（4：1）10.0 mlを加え，超音波処理（約25℃，20分間）し，遠心分離後，上清を試験溶液とした[6]．定量は2回行い，その平均値を求めた．

試験溶液の分析条件は既報[2,6]に従った．

結　果

1. 保存中のフロクマリンの経時変化

ヨロイグサの乾燥根粉末を種々の条件で1年間保存し，各々の条件におけるフロクマリン含量の経時変化を調べた．一例として，ガラス瓶に入れ温室内に放置（条件D）した際の経時変化をFig. 2に示す．なお，psoralen，xanthotoxin，bergapten，byak-angelicol，oxypeucedanin，imperatorin，phellopterin及びisoimperatorinの初期値は，それぞれ2.5，4.3，11.3，487，404，132，63及び85 mg/100 gであった．

主成分であるbyak-angelicolは時間の経過と共に減少し，1年後には約4分の1の含量を示した．同様にoxypeucedaninも減少したが，その度合いは前者よりも緩やかで，4カ月後には含量の逆転が認められた．含量のより少ないimperatorin，phellopterin及びisoimperatorinも時間の経過と共に減少した．ストレス化合物（ファイトアレキシン）であるpsoralen，xanthotoxin及びbergapten[3]はスケールの関係上Fig. 2から削除したが，上記の5成分と同様に時間の経過と共に減少した．

2. 保存後の残存率

6条件下における8成分の1年後の残存率をTable 1に示す．

冷暗所保存（A）では，8成分の残存率は95～100%という高い値を示した．室内放置の場合，ガラス瓶中（B）では70～86%，ポリ袋中（C）では52～83%の値が得られ，保存容器の違いにより残存率に差が認められた．温室保存の

場合，室内放置より更に大きい減少が認められ，一部の成分ではガラス瓶中（D）とポリ袋中（E）で残存率に差が認められた．ガラス瓶をアルミ箔で包んで遮光（F）すると，6成分の残存率は93%以上の高い値を示したが，byak-angelicolは68%，oxypeucedaninは75%の低い値を示した．

3. 紫外線照射による減少

紫外線照射1週間後の各成分の残存率をTable 2に示す．

直接照射（G）では大きく減少し，8成分の残存率は35～75%を示した．主成分であるbyak-angelicolの残存率は69%，oxypeucedaninは75%であった．他の6成分の残存率を比較すると，imperatorinが35%と最も低い値を示した．なお，ブランク（G'）の残存率は94～100%で，5成分に若干の減少が認められた．

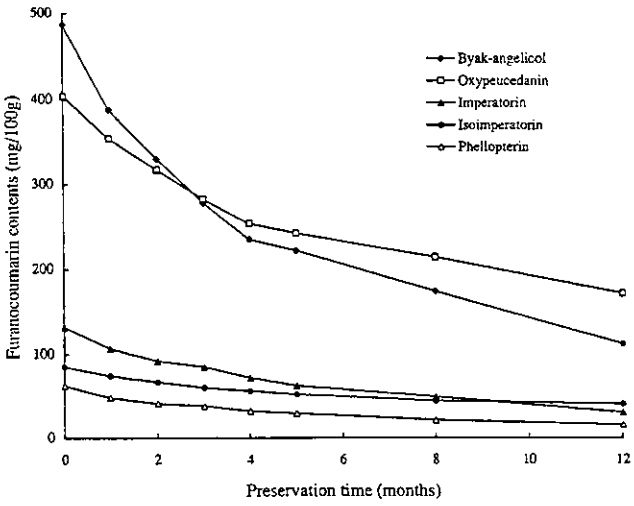

Fig. 2. Time-course Study of Eight Furanocoumarin Contents in Angelica dahurica Root during Preservation for One Year.
Powdered Angelica dahurica root in a glass centrifuge tube with a screw cap was placed in a greenhouse from June 1, 2001 to June 3, 2002 (condition D).

一方，ガラス瓶中（H）の残存率は75～97%であった．

このように条件GとHで大きな差が認められ，これは主として360 nm以下の波長の紫外線透過率の違いに起因し，本波長領域が減少に大きく関与していることが示唆された．

4. 加熱による減少

加熱1週間後の各成分の残存率をTable 3に示す．

40℃（I）では各々の残存率は90～100%の高い値を示した．50℃（J）では主成分であるbyak-angelicol及びoxypeucedaninの減少が認められ，各々の残存率は62%及び71%に低下した．60℃（K）ではbyak-angelicol及びoxypeucedaninは顕著に減少し，各々の残存率は10%以下であったが，imperatorin, phellopterin及びisoimperatorinは81～89%と高い値を示し，psoralen, xanthotoxin及びbergaptenでは100%と全く影響は認められなかった．

保存法別試料における遮光条件下温室内保存（F）の結果より，psoralen, xanthotoxin, bergapten, imperatorin, phellopterin及びisoimperatorinは熱に安定であるが，byak-angelicol及びoxypeucedaninは不安定であることが示唆され，本加熱実験において，この結果をほぼ裏付ける結果が得られた．

考　　察

長時間のケイ酸カラムクロマトグラフィーにより，byak-angelicolは開裂してbyak-angelicinに変化するが，oxypeucedaninは安定であることが報告されている[7]．従って，byak-angelicol及びoxypeucedaninはどちらも側鎖にエポキシ環を有するが，その安定性は異なるものと考えられる．今回の実験結果から，byak-angelicolはもとよりこれまで安定と考えられてきたoxypeucedaninも室温保存中に経時的に減少することが明らかになった．両者

Table 1. Recovery Ratios of Eight Furanocoumarins in Powdered Angelica dahurica Roots after Preservation under Various Conditions for One Year.

Preservation conditions				Recovery Ratios (%)							
Place/Temperature/Container				Pso	Xan	Ber	BAng	OPeu	Imp	Phe	iImp
A	Cold room	4.5℃	Glass	100	100	99	95	96	96	95	95
B	Laboratory	15～32℃	Glass	80	86	86	71	78	70	83	84
C	〃	〃	Vinyl	52	74	75	65	76	56	56	83
D	Greenhouse	6～58℃	Glass	20	35	37	23	42	23	26	49
E	〃	〃	Vinyl	16	30	28	24	40	22	23	34
F	〃	〃	Al foil	96	95	96	68	75	92	93	94

Recovery ratios were calculated from the value before preservation. The samples were preserved under each condition from June 1, 2001 to June 3, 2002.

Pso, psoralen; Xan, xanthotoxin; Ber, bergapten; BAng, byak-angelicol; OPeu, oxypeucedanin; Imp, imperatorin; Phe, phellopterin; iImp, isoimperatorin.

Container: glass, a centrifuge tube with a screw cap (50 ml); vinyl, a polyethylene bag; Al foil, a centrifuge tube with a screw cap (50 ml) wrapped in aluminum foil.

Table 2. Recovery Ratios of Eight Furanocoumarins in Powdered Angelica dahurica Roots after UV Irradiation under Two Conditions for One Week.

Irradiation condition	Recovery Ratios (%)							
	Pso	Xan	Ber	BAng	OPeu	Imp	Phe	iImp
Unshield								
G UV irradiation	50	50	52	69	75	35	42	60
G′ Blank	100	100	100	98	97	95	95	94
In glass bottle								
H UV irradiation	88	97	75	93	94	89	86	92
H′ Blank	100	100	100	100	100	100	100	100

Recovery ratios were calculated from the value before each treatment. Irradiation was done by a 15 W sterilizing UV lamp (GL15, SANKYO DENKI Co., Ltd.), which was set at twenty cm distance from the samples (9 J/m^2·sec) in a paper box at temperatures of 24 to 28 ℃.

Irradiation condition: Unshield, the sample was directly irradiated; In glass bottle, the sample was shielded in a centrifuge tube with a screw cap (TE-32, PYREX, IWAKI GLASS Co., Ltd.).

Other notes are the same as Table 1.

Table 3. Recovery Ratios of Eight Furanocoumarins in Powdered Angelica dahurica Roots after Heating at Various Temperatures for One Week.

Temperature	Recovery Ratios (%)							
	Pso	Xan	Ber	BAng	OPeu	Imp	Phe	iImp
I 40±0.5℃	100	100	100	90	92	97	100	98
J 50±0.5℃	100	100	100	62	71	94	94	96
K 60±0.5℃	100	100	100	7	9	81	84	89

Recovery ratios were calculated from the value before heat treatment. Heat treatment was carried out in a heating block (HL-21, YAMATO Co., Ltd.) with a paper lid in which a thermometer was inserted. The samples were shielded in a centrifuge tube with a screw cap (50 ml) wrapped in aluminum foil.

Other notes are the same as Table 1.

を比較すると，byak-angelicol の方がより低い残存率を示した．紫外線照射及び加熱実験の結果から，両者の減少の主原因は光及び熱による分解であると考えられた．

側鎖として 3,3-dimethylallyloxy 基を有する imperatorin, phellopterin 及び isoimperatorin は熱には比較的安定であったが，直射日光（紫外線）には不安定で，特に imperatorin 及び phellopterin の減少が著しかった．Fig. 1 に示す構造式の R^2 に側鎖を有する byak-angelicol, imperatorin 及び phellopterin は R^1 に各々同一の側鎖を有する oxypeucedanin 及び isoimperatorin と比較して，光及び熱に対してより低い残存率を示した．側鎖の種類及び位置による反応性の違いを反映しているものと推察される．

側鎖を有さない psoralen, xanthotoxin 及び bergapten は加熱及び遮光条件下では安定であったが，採光条件下では大きく減少し，日光あるいは蛍光灯下での残存率は psoralen で低い傾向にあった．これらの 3 化合物はいずれも人の皮膚に付いた後に日光に当たると皮膚炎を起こすことが知られており，psoralen の作用が最も強いとされている[8]．今回の psoralen の残存率の低さは，その光毒性（反応性）の強さを反映しているものと考える．

紫外線照射実験により，360 nm 以下の波長の紫外線が減少に関与していることが示唆されたが，リニア型フロクマリンは波長の長い 320～400 nm で強い光反応性を示し，280～315 nm では有意な光反応性を示さないことが知られている[9]．本実験において，すべてのフロクマリンが温室内保存 (D, E) において著しい減少がみられたのは，320～400 nm の紫外線を長期間浴びたことに起因すると考えられる．ガラス瓶保存 (B, D) とポリ袋保存 (C, E) における各化合物の残存率の差は，透過する紫外線の波長領域及びその透過率の違いに加え気体透過性及び透湿性の違いによると考察する．

以上のように，今回定量したフロクマリン 8 種はいずれも室温保存中に徐々に減少し，その減少率は側鎖の有無，側鎖の位置により大きく異なることが明らかになった．今後，白芷成分の研究を進める際には，実験材料の保存状態，保存期間などを考慮しなければならないと考える．

なお，白芷の調製法の違い（日照条件，温風仕上げの有

無）が成分含量に及ぼす影響について現在検討中であり，これらの結果については改めて報告する予定である．

ま と め

白芷の保存条件が，8種類のフロクマリンの含量に及ぼす影響を経時的に調べた．その結果，白芷を光の当たる室温下や温室に放置すると，psoralen, xanthotoxin, bergapten, byak-angelicol, oxypeucedanin, imperatorin, phellopterin 及び isoimperatorin は時間の経過と共にいずれも減少したが，冷暗所保存では減少が認められなかった．

この原因を明らかにするため，1週間の紫外線照射試験ならびに 40～60℃ の加熱試験を行った結果，主成分である byak-angelicol 及び oxypeucedanin は紫外線照射により減少し，50℃ 以上の加熱により著しく減少した．他の6成分も紫外線照射により減少したが，加熱に対しては比較的安定であった．

以上の結果から，白芷中のフロクマリンは保存中の温度や光，特に紫外線の影響を受けやすいことが明らかになった．

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Molecular cloning and characterization of a glucosyltransferase catalyzing glucosylation of curcumin in cultured *Catharanthus roseus* cells

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Abstract ***Catharanthus roseus*** **cell suspension cultures are capable of converting exogenously supplied curcumin to various glucosides. The glucosylation efficiency is enhanced by addition of methyl jasmonate (MJ) to the cultures prior to curcumin administration. Two cDNAs encoding UDP-glucosyltransferases (CaUGT1 and CaUGT2) were isolated from a cDNA library of cultured *C. roseus* cells, using a PCR method directed at the conserved UDP-binding domain of plant glycosyltransferases. The sequence identity between their deduced amino acid sequences was 27%. The expression of both genes was up-regulated by addition of MJ to the cell cultures although the mRNA level of CaUGT1 was much lower than that of CaUGT2. The corresponding cDNAs were expressed in *Escherichia coli* as fusion proteins with maltose-binding protein. The recombinant CaUGT1 exhibited no glucosylation activity with either curcumin or curcumin monoglucoside as substrate, whereas the recombinant CaUGT2 catalyzed the formation of curcumin monoglucoside from curcumin and also conversion of curcumin monoglucoside to curcumin diglucoside. The use of the recombinant CaUGT2 may provide a useful new route for the production of curcumin glucosides.**

Keywords: Glucosyltransferase; Curcumin; Heterologous expression; Substrate specificity; Cell suspension culture; *Catharanthus roseus*

1. Introduction

A remarkably diverse array of glycosyltransferases is present in plant cells as exemplified by more than 110 glycosyltransferase genes in the *Arabidopsis* genome [1,2]. These glycosyltransferases are involved not only in biosynthesis of natural products but also in regulation of the cellular level of plant hormones and signal molecules, and in detoxification of xenobiotics [3]. Some glycosyltransferases have been shown to exhibit broad substrate specificity [4] which could enable them to be used in enzyme-catalyzed transfer of sugars to aglycones as an alternative approach to chemical synthesis of useful glycosides [5].

Curcumin is a yellow pigment of turmeric (dried rhizome of *Curcuma longa*, Zingiberaceae). It has been used primarily as a food colorant, but it is also a pharmacologically active principle of turmeric which has been used as a traditional medicine. Curcumin has recently attracted increased attention because of its potent anti-inflammatory and anti-leishmanial properties [6], and ability to reduce alcohol-induced liver disease [7]. Curcumin has also been reported to exhibit anti-cancer activity, based on various molecular mechanisms [8]. Furthermore, curcumin has been shown to suppress amyloid β-protein (Aβ)-induced oxidative damage and to prevent Aβ-infusion-induced spatial memory deficits in rats, suggesting a possible clinical application for treatment of Alzheimer's disease [9]. Although curcumin is an interesting compound as a novel medicine or a lead compound, its low water solubility limits further pharmacological exploration and practical application.

We previously indicated that *Catharanthus roseus* cell suspension cultures converted exogenously supplied curcumin to a series of glucosides [10]. The water solubility of curcumin glucosides is increased up to 20-million-fold compared with that of the aglycone, indicating that glucosylation is a powerful method to enhance curcumin water solubility. Furthermore, bioavailability of curcumin may be increased by glucosylation. It has been reported that, while the majority of orally administered curcumin in rats was excreted in the feces without being absorbed, some portion entered blood circulation after conjugation with glucuronic acid in the intestinal mucosa [11]. However, the glucosylation efficiency of cultured *C. roseus* cells was relatively low.

In the present paper, we describe the isolation of two cDNAs encoding novel glucosyltransferases, designated CaUGT1 and CaUGT2 (*Catharanthus roseus* UDP-glucose glucosyltransferase), from *C. roseus* cultured cells. Functional expression of the gene products in *Escherichia coli* demonstrated that CaUGT2 converted curcumin to curcumin monoglucoside and curcumin diglucoside. Recombinant CaUGT2 enzyme thus provides us with an efficient method to produce curcumin glucosides from curcumin.

2. Materials and methods

2.1. Cell cultures

Catharanthus roseus cell suspension cultures were maintained in LS medium [12] supplemented with 3% sucrose, 1 μM 2,4-dichlorophen-

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Abbreviations: CaUGT, *Catharanthus roseus* UDP-glucosyltransferase; IPTG, isopropyl 1-thio-β-D-galactoside; LS, Linsmaier and Skoog; MJ, methyl jasmonate; Pfu, plaque forming unit; PSPG, plant secondary product glycosyltransferase

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oxyacetic acid and 1 μM kinetin. The cells were cultured at 25 °C in the dark and subcultured at 2-week-intervals. Methyl jasmonate (MJ) was dissolved in dimethylsulfoxide and aseptically added to the cultures through membrane filters at a final concentration of 250 μM three days after cell inoculation. The cells were collected by vacuum filtration at the defined times, immediately frozen in liquid nitrogen, and stored at −75 °C until use.

2.2. Construction of cDNA library

Poly(A)$^+$RNA was prepared from *C. roseus* cells harvested 24 h after MJ addition, using a Quick Prep mRNA Purification Kit (Amersham). The cDNA library was constructed using a λZAP cDNA Synthesis/Gigapack Gold Packaging Kit (Stratagene).

2.3. PCR cloning of glucosyltransferase cDNAs

A 5′-sense degenerate primer (5′-TT(T/C)(T/C/G)TI(A/T)(G/C) ICA(T/C)TG(T/C)GGITGGAA) was designed based on the amino acid sequence F(L/V)(T/S)HCGWN in the conserved PSPG-box of plant glucosyltransferases [13]. A 1 μl aliquot (33 000 pfu) of the cDNA library was used as a template for PCR amplification in a 25 μl reaction mixture containing 0.2 mM dNTP, 100 nM degenerate primer, 25 nM T7 primer and 0.5 unit *Taq* Polymerase (Roche Biochemicals). PCR was carried out using the following parameters; denaturing at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 40 °C for 2 min and 72 °C for 1 min, and then a final extension at 72 °C for 10 min. PCR products of about 500 bp were recovered from an agarose gel and subcloned into pCR2.1-TOPO vector (Invitrogen). Randomly selected clone inserts were sequenced for both strands using the Thermo Sequenase Cycle Sequencing Kit (Amersham). This yielded nine clones whose sequences fell into two independent sequence classes, both of which displayed significant similarity with various plant glucosyltransferases. The 5′-fragment was obtained by PCR using a common forward primer of the T3 sequence and reverse primers specific to the 3′-fragments and sequenced. Finally, using the 5′ and 3′ sequences as specific primers the near full-length cDNA clones were amplified from the cDNA library.

2.4. Heterologous expression in E. coli

For construction of the pMAL-CaUGT1 and pMAL-CaUGT2 expression vectors, the open reading frames of CaUGT1 and CaUGT2 were amplified by PCR with primers 5′-GCGCGAATTCATGGAAGAGATGAAGAAAGT and 5′-GCGCAAGCTTCTCATGATATAGTTTTCTTC for CaUGT1 and 5′-GCGCGGATCCATGGTTAATCAGCTCCATAT and 5′-GCGCGTCGACCCTAGTCTTGTTGCTTTCTT for CaUGT2. These primers correspond to the 5′-and 3′-ends of the open reading frames and include appropriate restriction sites (underlined). The 1.5 kb PCR products were subcloned into the pMAL-c2 vector (New England Biolabs) and sequenced to ensure that no mutation was incorporated. The resulting expression vectors pMAL-CaUGT1 and pMAL-CaUGT2 were used to transform *E. coli* JM109. The transformed bacteria were cultured at 37 °C in Luria–Bertani medium containing 100 μg/ml carbenicillin until they reached OD_{600} values of about 1.0, and then isopropyl 1-thio-β-D-galactoside (IPTG) was added to the culture at a final concentration of 1 mM. The induced bacteria were further cultured at 30 °C overnight and then harvested by centrifugation. Preparation and purification of the enzymes were performed at 4 °C. The crude recombinant enzyme was obtained by sonicating the bacterial pellet in 20 mM Tris–HCl buffer (pH 7.6) containing 200 mM NaCl and 5 mM EDTA followed by centrifugation at 12 000 × *g* for 15 min at 4 °C. The soluble fraction was applied to an Amylose Resin (New England Biolabs) column (bed volume, 2.0 ml). After the column was washed with column buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl and 1 mM EDTA), the protein was eluted with 20 mM maltose in the column buffer. Protein content in the enzyme preparations was estimated using the method of Bradford [14].

2.5. Enzyme assays

For enzymatic assays of CaUGT1 and CaUGT2, 100 μl of a standard reaction mixture containing 50 mM Tris–HCl buffer (pH 7.5), 250 μM phenolic substrate, 2 mM UDP-glucose and the crude enzyme preparation (0.2 mg protein) was incubated at 30 °C for 30 min. The reaction was terminated by adding 200 μl methanol. After centrifugation at 12 000 × *g* for 10 min, the reaction products were analyzed by high performance liquid chromatography (HPLC). For determination of kinetic parameters of CaUGT2, the concentrations of the phenolic substrates were varied from 25 μM to 1 mM for curcumin and curcumin monoglucoside, and from 100 μM to 2.5 mM for other phenolics, in the presence of 2 mM UDP-glucose. For determination of K_m and V_{max} values for UDP-sugars, their concentrations were varied from 250 μM to 2 mM with the concentration of curcumin maintained at 250 μM. The incubations were carried out at 30 °C for 5 min. The kinetic values were estimated from Lineweaver–Burk plots from duplicate experiments.

HPLC analysis was performed on a reverse phase column (COSMOSIL 5C18-ARII, Nacalai Tesque) and the eluates were monitored by a photodiode array detector. The solvent conditions for separation of curcumin and curcumin glucosides were as described previously [10]. For separation of the other phenolic substrates and their glucosides, the following gradient elutions were used (flow rate 1.0 ml/min):

For esculetin, scopoletin and their glucosides: 0–8 min, 0–35% methanol; 8–9 min, 35–55% methanol; and 9–12 min, 55–100% methanol.

For *p*-nitrophenol, vanillin and their glucosides: 0–8 min, 20–55% methanol; 8–9 min, 55–100% methanol; and 9–12 min, 100% methanol.

For capsaicin, [6]-gingerol and their glucosides: 0–14 min, 40–79% methanol; 14–15 min, 79–100% methanol; and 15–20 min, 100% methanol.

The identity of the products was determined by co-chromatography on HPLC with the standard compounds and by their ultra-violet absorption spectra. The amounts of the products were determined based on the standard curves.

Curcumin, UDP-glucose and UDP-galactose were purchased from Sigma. Esculetin, esculin, scopoletin, *p*-nitrophenol and capsaicin were obtained from Wako Pure Chemicals. Vanillin and *p*-nitrophenyl-β-D-glucoside were from Nacalai Tesque. Glucovanillin was from our laboratory stocks. Curcumin monoglucoside and curcumin diglucoside were generous gifts of Profs. K. Isobe and K. Mohri of Showa Pharmaceutical University.

2.6. Analysis of gene expression by northern hybridization

Total RNA was prepared from the cultured cells using Trizol (Invitrogen). For northern hybridization, total RNA (10 μg) was electrophoresed in a formamide-containing agarose gel and blotted onto Hybond N+ (Amersham). Hybridization with digoxigenin-labeled probes and chemiluminescent detection were carried out according to a protocol supplied by Roche Biochemicals.

3. Results

3.1. Isolation of UDP-glucosyltransferase cDNAs by PCR from C. roseus *cDNA library*

Products resulting from PCR using a degenerate primer designed based on the highly conserved amino acid sequence among plant glycosyltransferases and a 3′-non-specific antisense primer (T7 primer) complementary to a λZAP II vector sequence displayed a band of about 500 bp, which was subcloned into a pCR2.1-TOPO vector. Among 30 clones randomly sequenced, 15 were found by database search to be similar to various plant glucosyltransferases. These clones were classified into two different groups (Ca-1 and Ca-2).

Full-length clones corresponding to both groups were isolated by 5′-rapid amplification of cDNA ends by using a 5′-non-specific sense primer (T3 primer) complementary to a λZAP-II vector sequence and sequence-specific antisense primers complementary to Ca-1 or Ca-2 sequence. The resulting clones were designated CaUGT1 and CaUGT2. The nucleotide sequences of CaUGT1 and CaUGT2 have been deposited in the DDBJ/EMBL/GenBank Nucleotide Sequence Database under the accession numbers AB159212 and AB159213, respectively.

CaUGT1 (1669 nucleotides) contains an open reading frame corresponding to a protein of 480 amino acids, whereas CaUGT2 (1722 nucleotides) has an open reading frame for 487

amino acids. The sequence identity between CaUGT1 and CaUGT2 was 27% on an amino acid base. The amino acid sequence of CaUGT1 revealed 55% identity with two tobacco glucosyltransferases (Nt1a and Nt1b) [4]. CaUGT2 was 62% identical with a salicylate-induced glucosyltransferase (IS5a) [15] and a phenylpropanoid glucosyltransferase of tobacco [16], and 61% identical with an anthocyanin 3′-glucosyltransferase of *Gentiana triflora* [17].

3.2. *Bacterial expression of CaUGT1 and CaUGT2 cDNAs and properties of the recombinant enzymes*

To examine the catalytic function of CaUGT1 and CaUGT2, both genes were expressed in *E. coli* as fusions with maltose binding protein and the crude enzyme extract was used for enzyme activity assays using either curcumin or curcumin monoglucoside as an acceptor substrate in the presence of UDP-glucose. As shown in Fig. 1A, the crude enzyme containing recombinant CaUGT2 converted both curcumin (**3**) and curcumin monoglucoside (**2**) to curcumin monoglucoside and curcumin diglucoside (**1**), respectively. Both reactions were completely dependent on UDP-glucose. In contrast, no products were detected when the recombinant CaUGT1 was incubated with either curcumin or curcumin monoglucoside. The crude enzyme preparation from the IPTG-induced bacteria harboring the control vector pMAL-c2 produced no glucosylation products from either curcumin or curcumin monoglucoside. The time-course pattern of the glucosylation reaction with curcumin (Fig. 1B) indicated that curcumin was first converted to curcumin monoglucoside which was then further glucosylated to produce curcumin diglucoside by the single enzyme of the recombinant CaUGT2. After 120 min incubation, the complete conversion of curcumin into the diglucoside was achieved.

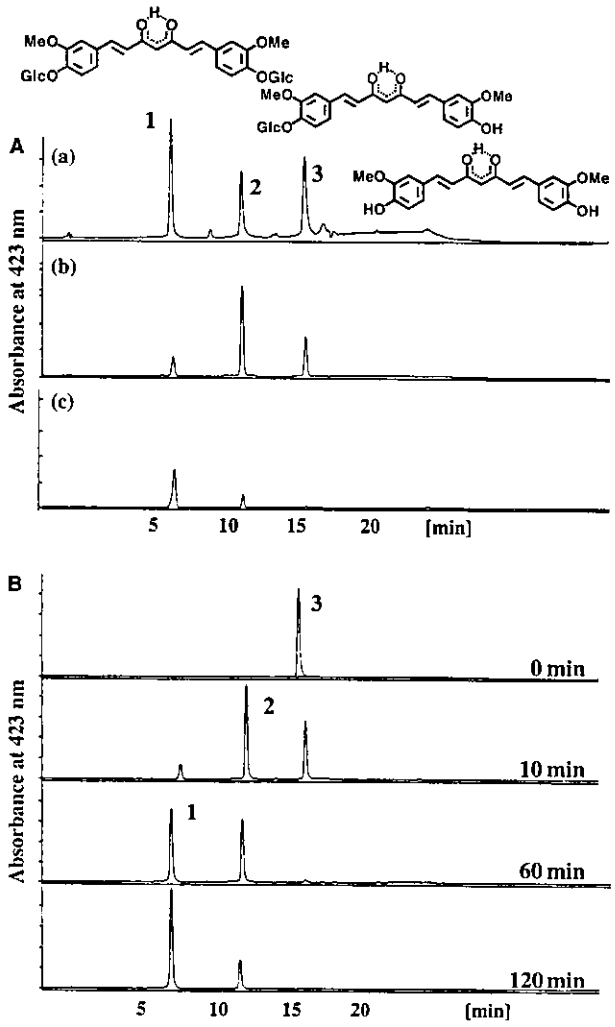

Fig. 1. Analysis of glucosyltransferase activity of recombinant CaUGT2 expressed in *E. coli*. (A) HPLC chromatogram of authentic standards of curcumin diglucoside (**1**, R_t = 7.4 min), curcumin monoglucoside (**2**, R_t = 11.8 min) and curcumin (**3**, R_t = 15.8 min) (a); The enzyme assay was carried out with the crude protein prepared from *E. coli* expressing recombinant CaUGT2 using curcumin (b) or curcumin monoglucoside (c) as a substrate. The eluate was monitored at $UV_{423\,nm}$. The identity of the reaction products was confirmed by comparison of the retention time and UV-spectrum (recorded on a diode array detector) with those of authentic standards. (B) Time course of changes in glucosylation of curcumin by incubation with the crude enzyme from *E. coli* producing recombinant CaUGT2. Curcumin was incubated for 0, 10, 60 and 120 min at 30 °C with 0.16 mg crude protein. The eluate was monitored at 423 nm.

To analyze the biochemical properties of CaUGT2, the recombinant fusion protein was purified by affinity chromatography using Amylose Resin (Fig. 2). The pH optimum of the enzyme was 7.5–8, and the glucosylation reaction proceeded linearly over a 10-min incubation and then the rate was gradually decreased under the present assay conditions. By using the affinity-purified fusion protein, substrate specificity for various phenolic compounds (Fig. 3) was examined as shown in Table 1. Apparent K_m-values for curcumin and curcumin monoglucoside were determined to be 19 and 63 μM, respectively. Apparent K_m-values for coumarins, *p*-nitrophenol and vanillin were more than 10-fold higher than those of curcumin and curcumin monoglucosides (Table 1). The V_{max}/K_m ratios indicated that CaUGT2 exhibits the highest specificity towards curcumin (100%), followed by curcumin monoglucoside (64%), esculetin (38%), scopoletin (10%),

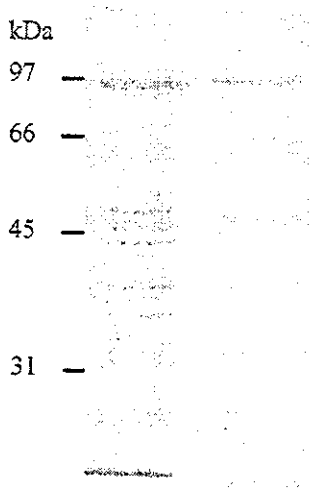

Fig. 2. SDS–PAGE analysis of the crude protein prepared from IPTG-induced *E. coli* harboring pMAL-CaUGT2 and fusion protein of recombinant CaUGT2 purified using amyrose-resin column. The crude protein (11 μg; left) and the purified protein (1 μg; right) were separated by 10% (w/v) SDS–PAGE gel and visualized with Coomassie Brilliant Blue staining.

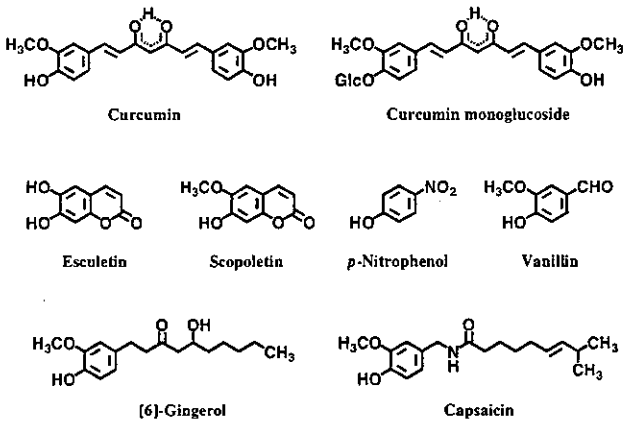

Fig. 3. Chemical structures of phenolic compounds used as glucosyl acceptors in the present investigation.

Table 1
Kinetic parameters of recombinant CaUGT2 towards various phenolic substrates

Substrates	K_m (μM)	V_{max} (pkat/mg)	V_{max}/K_m
Curcumin	19.0	273	14.9
Curcumin mono-glucoside	63.3	586	9.25
Esculetin	660	3634	5.50
Scopoletin	1120	1584	1.41
p-Nitrophenol	836	452	0.54
Vanillin	993	212	0.21
UDP-glucose	124	234	1.88
UDP-galactose	501	39.8	0.080

p-nitrophenol (4%) and vanillin (2%). In contrast, neither [6]-gingerol nor capsaicin was glucosylated although their chemical structures are somewhat similar to curcumin. The apparent K_m-values of sugar donors were 124 μM for UDP-glucose and 501 μM for UDP-galactose (Table 1).

We also examined glucosylation of the above mentioned phenolic compounds and two flavonols, kaempferol and luteolin, by crude recombinant CaUGT1 but none of these substrates was converted to the corresponding glucosides.

3.3. Methyl jasmonate-induced gene expression

It was shown previously that glucosylation of curcumin by cultured *C. roseus* cells was enhanced by addition of MJ to the cultures [10]. The effects of MJ on the mRNA level of CaUGT1 and CaUGT2 were therefore examined. As shown in Fig. 4, the expression of CaUGT2 was rapidly induced by addition of MJ to the cultures. Although the expression level of CaUGT1 was too low to be detected by northern hybridization, the CaUGT1 mRNA accumulation was also increased by MJ addition, when estimated by RT-PCR (data not shown).

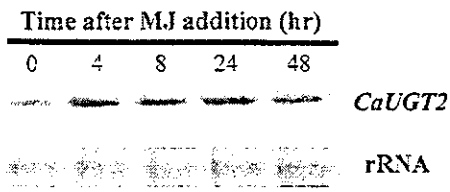

Fig. 4. Northern blot analysis of mRNA levels of CaUGT2 in *C. roseus* cell suspension cultures after the addition of MJ. The suspension cultures were supplemented with MJ at a final concentration of 250 μM three days after cell inoculation and the cells were collected for analysis 0, 4, 8, 24 and 48 h after MJ addition.

4. Discussion

Plant glycosyltransferases comprise a super-family of plant genes similar in magnitude to the *O*-methyltransferase [18] and cytochrome P450 [19] gene families. These enzyme classes are all extensively involved in modification of secondary metabolites, thus contributing to the diversity of plant chemistry. Based on the multiple alignment of a set of putative glycosyltransferases of *Cassava* and several glycosyltransferases mainly involved in flavonoid metabolism, a consensus sequence consisting of a short stretch of about 40 amino acids close to the C-terminal part of the protein has been proposed as a conserved domain for glycosyltransferases involved in plant secondary metabolism [20]. Within this PSPG-box, a peptide sequence of HCGWNS has been detected in 95% of all glycosyltransferases that catalyze inversion of the anomeric bond from the α-linkage found in UDP-sugar to the β-configuration found in the resulting glycosides [13]. By using a degenerate primer designed to target this peptide sequence, we isolated two cDNAs (CaUGT1 and CaUGT2) which encode putative glucosyltransferases from a cDNA library of cultured *C. roseus* cells.

The molecular phylogenetic tree (Fig. 5) based on a multiple sequence alignment of CaUGT1, CaUGT2 and 17 other plant UDP-glycosyltransferases retrieved from the database showed that CaUGT1 is placed adjacent to glucosyltransferases Nt1a and Nt1b of tobacco [4] and arbutin synthase of *Rauvolfia serpentina* [5]. All of these glucosyltransferases exhibit wide substrate specificity and catalyze glucosylation of various xenobiotic phenols such as naphthols, scopoletin and hydroquinone. CaUGT2, on the other hand, belongs to a cluster containing diverse glucosyltransferases such as flavonoid 7-glucosyltransferase of *Scutellaria baicalensis* [21], betanidin 5-glucosyltransferase of *Dorotheanthus bellidiformis* [22], flavonoid 3′-glucosyltransferase of *Gentiana triflora* [17] and *Arabidopsis* UGT73C6 [23]. The *Gentiana* and *Arabidopsis* enzymes catalyze transfer of glucose to the 3′-position of delphinidin 3, 5-*O*-diglucoside and 7-position of kaempferol 3-*O*-rhamnoside, respectively. Based on the relation of the dendrogram clusters to reported substrate specificities, we expected that CaUGT1 would be a likely candidate for catalyzing glucosylation of curcumin.

Heterologous expression experiments clearly showed, however, that only CaUGT2 was able to glucosylate curcumin, and that it did so in a sequential manner, converting curcumin to curcumin monoglucoside and then curcumin monoglucoside to curcumin diglucoside. CaUGT1, on the other hand, glucosylated neither curcumin nor curcumin monoglucoside. We have not found an efficient substrate for CaUGT1 among the various natural and unnatural phenolic compounds so far examined. The substrate specificity of CaUGT2, which was tested on a wide range of phenolic substrates, was relatively broad with the highest affinity for curcumin followed by curcumin monoglucoside and esculetin although it did not glucosylate [6]-gingerol or capsaicin, both of which show structural similarities to curcumin. Ferulic acid, which also has a similar

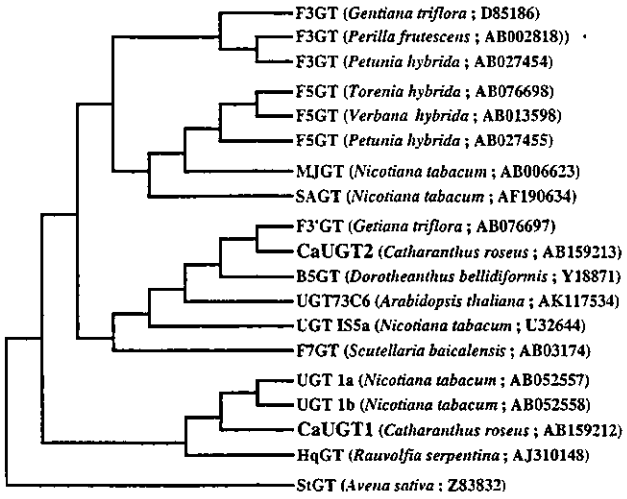

Fig. 5. Phylogenetic tree showing the relationship between two UDP-glucosyltransferases from *C. roseus* and some other plant secondary product glucosyltransferases retrieved from the database. The amino acid sequences were aligned using Clustal X and the tree was constructed by the neighbor-joining method in PHYLIP software package (J. Felsenstein, Phylogeny Inference Package, version 3.573, University of Washington, Seattle, WA, USA). Applying the parsimony-based method gave essentially the same topology. F3'H, flavonoid 3'-*O*-glucosyltransferase; F3H, flavonoid 3-*O*-glucosyltransferase; F5H, flavonoid 5-*O*-glucosyltransferase; MJGT, jasmonate-induced glucosyltransferase; SAGT, salicylic acid glucosyltransferase; F7GT, flavonoid 7-*O*-glucosyltransferase; B5GT, betanidin 5-*O*-glucosyltransferase; UGT1a, tobacco glucosyltransferase Nt1a; UGT1b, tobacco glucosyltransferase Nt1b; HqGT, hydroquinone *O*-glucosyltransferase; StGT, sterol *O*-glucosyltransferase. The species names of the original plants and database accession numbers are indicated in parentheses.

structure to curcumin, was glucosylated to some extent but further kinetic analysis was not performed because authentic ferulic acid glucoside was not available. The kinetic constants of recombinant CaUGT2 towards various phenolic substrates were within the same range displayed by other phenolic glucosyltransferases so far examined. Both UDP-glucose and UDP-galactose could support the glycosylation of curcumin by CaUGT2 although apparent K_m-value for UDP-galactose was 4-fold higher than that for UDP-glucose and the V_{max}/K_m ratio was only 4% of that for UDP-glucose, a pattern analogous to that reported for the tobacco glucosyltransferase Nt1a, which preferred UDP-glucose but also utilized UDP-xylose [3].

It is interesting to note that although esculetin was efficiently glucosylated by CaUGT2, the sole product was esculetin 6-*O*-β-monoglucoside (esculin); i.e., formation of esculetin 6,7-*O*-β-diglucoside was not detected. This indicates that conjugation of a glucose molecule to one phenolic hydroxyl group of curcumin does not create steric hindrance for the second glucosylation, whereas a glucosyl residue attached to 6-hydroxyl position of esculetin may interfere with further glucosyl conjugation to the neighboring 7-hydroxyl group. The result also suggests that CaUGT2 might have a strict position specificity for the hydroxyl group it can act upon.

Northern hybridization analysis indicated that expression of CaUGT2 was rapidly up-regulated by addition of MJ to the cells. This is consistent with our previous result [10] that glucosylation of curcumin in the cultured cells of *C. roseus* was markedly enhanced by adding MJ to the cell suspension cultures prior to curcumin addition, and may suggest, together with its relatively broad substrate specificity for both acceptor and donor molecules, that CaUGT2 is a defense-related UDP-glucosyltransferase whose function is detoxification of xenobiotic phenols or biosynthesis of defense molecules.

There have been some reports describing chemical synthesis of curcumin glucosides. Direct glucosylation of curcumin by acetobromoglucose followed by deacetylation gave curcumin monoglucoside and curcumin diglucoside at yields of 8% and 3%, respectively [24]. Recently, more efficient routes to curcumin monoglucoside and curcumin diglucoside starting from vanillin were reported with total yields of 35% and 21%, respectively [25]. In contrast to these, incubation of 1 μmole curcumin and 20 μmole UDP-glucose in the presence of 4 mg crude bacterial protein containing recombinant CaUGT2 in a total volume of 1 ml for 3 h at 30 °C yielded 735 nmole curcumin diglucoside, corresponding to 74% yield, without tedious purification of synthetic intermediates or any need for protection and deprotection of reactive groups. In a preliminary experiment, we also examined in vivo glucosylation by using intact recombinant *E. coli* cells expressing CaUGT2, in order to circumvent the use of relatively expensive UDP-glucose in the in vitro synthesis. However, the glucosylation yield was 4% at maximum and the sole product was curcumin monoglucoside.

In conclusion, whatever the physiological function of CaUGT2 may be, the use of recombinant CaUGT2 may provide an efficient tool for the production of pharmacologically useful curcumin glucosides. Furthermore, integration and expression of CaUGT2 gene may result in an efficient biotransformation of this and other natural products in planta.

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Tropane Alkaloid Production and Shoot Regeneration in Hairy and Adventitious Root Cultures of *Duboisia myoporoides–D. leichhardtii* Hybrid

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Co-culture conditions for *Duboisia myoporoides–D. leichhardtii* hybrid hairy root induction were investigated using leaf explants and *Agrobacterium rhizogenes* ATCC 15834. The bacteria density and duration of co-culture greatly affected the induction rate; the highest rate of 50% was obtained when the leaf explants were co-cultured for 2 d with 10^6 bacteria. One hairy root clone that showed the fastest root growth was selected and used for comparison study with adventitious roots cultured with 0.5 mg/l indole-3-acetic acid (IAA). The hairy roots cultured in Murashige and Skoog (MS) liquid medium grew well and yielded much more tropane alkaloids (35 mg/l scopolamine and 17 mg/l hyoscyamine) than adventitious roots cultured in 0.5 mg/l IAA after 6 weeks of culture at 25 °C in the dark. The hairy and adventitious roots (2.5 cm) grown in liquid media were divided into 5 parts (each 0.5 cm) along the root axis. Distribution of scopolamine and IAA was then determined by enzyme-linked immunosorbent assay (ELISA). Inverse relationship between contents of scopolamine and IAA was observed in the hairy roots; increase of scopolamine and decrease of IAA were proportional to the distance from the root meristem. In contrast, the contents of scopolamine and IAA were relatively constant in the adventitious roots. In shoot regeneration experiments, the hairy and adventitious root segments (1 cm) were placed onto 1/2 MS solid medium containing various concentrations of IAA and BA cultured at 25 °C under 16 h light. In adventitious roots, the shoots regenerated on media containing 6-benzyladenine (BA) (0.5 to 5 mg/l), and 100% regeneration was observed in medium with 0.1 mg/l IAA and 2 mg/l BA. On the other hand, shoot regeneration was only observed in 33% of hairy roots cultured on medium containing 5 mg/l BA.

Key words *Duboisia* hybrid; hairy root; adventitious root; tropane alkaloid

Genus *Duboisia* (Family: Solanaceae) comprises four species, *Duboisia leichhardtii* F. MUELL, *D. myoporoides* R. BR., *D. hapwoodii* F. MUELL and recently discovered *D. arentitensis* L. A. CRAVEN, B. J. LEPSCHI & L. A. R. HAEGI. The leaves of *D. leichhardtii* and *D. myoporoides*, as well as their interspecific hybrid, are the principal source for pharmaceutically important tropane alkaloids such as scopolamine and hyoscyamine.[1] These alkaloids have anticholinergic property and, together with their semi-synthetic derivatives, are extensively used as spasmolytic and mydriatic agents.[2] Because scopolamine prominently depresses the central nervous system at low therapeutic doses, it is frequently administered for the prevention of motion sickness.[2] Cultivation study of *Duboisia* hybrid in Australia revealed that alkaloid contents change seasonally and decrease of scopolamine content occurred between March and September.[3] Regional variation of alkaloid composition and content has also been reported.[1]

To produce tropane alkaloids more efficiently and stably, a number of *in vitro* studies have been performed so far, and *Agrobacterium*-transformed hairy roots have been found to be suitable for the production of secondary metabolites because of their stable and high productivity in culture conditions without plant growth regulators.[4] There are several researches on the influences of T-DNA expression on secondary metabolism.[5] However, there are few reports on the relationship between endogenous auxin and alkaloids or shoot regeneration in the hairy roots. In our ongoing study on tropane alkaloid production by tissue cultures, we established hairy and adventitious root cultures of *Duboisia myoporoides–D. leichhardtii* hybrid. This work reports the formation of tropane alkaloids and the regeneration of shoots in hairy and adventitious root cultures of *Duboisia* hybrid. In addition, distribution of indole-3-acetic acid (IAA) and scopolamine in the hairy roots and in the adventitious roots is described.

MATERIALS AND METHODS

Plant Material Explants were excised from a *Duboisia myoporoides–D. leichhardtii* hybrid (M-II-8-6) that is cultivated at the Tsukuba Medicinal Plant Research Station, Japan. The leaves were dipped in 75% ethanol for 10 s, rinsed in sterilized water, surface-sterilized for 10 min in 2% sodium hypochlorite containing Tween 20 (1 drop per 40 ml) and then washed three times with sterilized water. The leaf segments (*ca.* 5×5 mm) were used for hairy and adventitious roots induction.

Hairy Root Culture The leaf segments were transferred into a half strength Murashige and Skoog[6] (1/2 MS) liquid medium containing 2% sucrose (20 ml medium in a 100 ml Erlenmeyer flask). *Agrobacterium rhizogenes* ATCC15834 was cultured overnight in Yeast Extract Broth (YEB)[7] liquid

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medium (20 ml medium in a 100 ml Erlenmeyer flask) and a portion (final bacteria densities were set as 10^5, 10^6 or 10^7) was added to the flask with leaf segments. They were co-cultured for 1 to 3 d at 25 °C in the dark on a rotary shaker (100 rpm). The segments (5 segments/dish, 3 dishes per treatment) were placed onto 1/2 MS solid medium (7 ml medium in 6 cm i.d. petri dish) containing 500 mg/l Claforan® (Hoechst Japan Ltd.) and 2% sucrose at 25 °C in the dark. The roots regenerated from the leaf segments were excised and repeatedly cultured on the same medium until all the bacteria were eliminated. After 3 weeks from the co-culture with bacteria, survival rate of the leaf explants, frequency of hairy root formation and number of hairy roots per explant were determined. The roots were further cultured on a Murashige and Skoog (MS) solid (25 ml in a 9 cm i.d. petri dish) and liquid (50 ml in a 100 ml Erlenmeyer flask) media containing 3% sucrose at 25 °C in the dark. Transformation was confirmed by opine analysis according to the method described by Petit *et al.*[8] The roots that grew fastest among the clones were selected and used for further experiments. The transformed roots (inoculum: 30 mg fresh weight) grown on MS solid medium were excised and inoculated into either MS, Gamborg B5 (B5),[9] Woody Plant (WP)[10] or White (WH)[11] liquid medium.

Adventitious Root Cultures The adventitious root cultures were established from the leaf explants as reported previously.[12] They were maintained in MS liquid medium containing 0.5 mg/l IAA with subculturing carried out at 4 to 8 weeks interval.

Shoot Regeneration The hairy and adventitious root segments (*ca.* 0.5 cm including root meristem) excised from the liquid cultures were placed onto 1/2 MS solid medium (25 ml in a 9 cm i.d. petri dish) supplemented with various concentrations of IAA and 6-benzyladenine (BA), and cultured under 16 h light. The regenerated shoots were transferred onto MS solid medium (30 ml in a 3 cm i.d.×15 cm test tube) and cultured under 16 h light.

General Culture Conditions All the liquid cultures were maintained on a rotary shaker at 100 rpm in the dark at 25 °C. The solid medium was solidified with 0.2% Gelrite. All the media contained 3% sucrose unless otherwise stated, and the pH was adjusted to 5.7 before autoclaving at 121 °C for 15 min. The light intensity was 80 $\mu E\,m^{-2}\,S^{-1}$.

Analysis of Tropane Alkaloids The hairy roots were lyophilized and ground prior to extraction. The culture medium was filtered prior to extraction. Alkaloids were extracted and analyzed by HPLC as previously reported.[12]

Determination of IAA and Scopolamine by ELISA The hairy roots were cultured in MS liquid medium and the adventitious roots were cultured in MS liquid medium containing 0.5 mg/l IAA at 25 °C in the dark. After 4 weeks, the fresh hairy and adventitious roots (2.5 cm in length) were thoroughly washed with water and divided into 5 parts (each 0.5 cm) from the distal to the proximal segment along the root axis. Each segment was subjected to enzyme-linked immunosorbent assay (ELISA) to determine IAA and scopolamine contents. Extraction and determination of IAA were carried out as previously reported[13] using ELISA kits for IAA (PHYTODETEK™-IAA). ELISA for scopolamine was performed using an anti-scopolamine monoclonal antibody according to the method described previously.[14] The cross-reactivities (%) of the anti-scopolamine antibody used for this study were 9.2 for 6β-hydroxyhyoscyamine, 8.2 for 7β-hydroxyhyoscyamine and 1.8 for hyoscyamine.[14]

RESULTS AND DISCUSSION

Hairy Root Induction Although there are several reports on hairy root cultures of *Duboisia* hybrid,[5,15,16] precise condition for hairy root induction such as bacteria density was not described. In our preliminary experiment, hairy roots of *Duboisia* hybrid with satisfactory growth could not be obtained from stem explants by the direct infection method previously reported.[15] Therefore co-culture method was employed, and the influence of bacteria density and duration of co-culture on hairy root induction was investigated using leaf explants and *Agrobacterium rhizogenes* ATCC 15834 (Figs. 1A, 2). The bacteria concentration and duration of co-culture greatly affected the induction rate; the highest rate of 50% was obtained when the leaf explants were co-cultured for 2 d with 10^6 bacteria (Fig. 2B). In this condition, two hairy root clones per explant were obtained (Fig. 2C). Celma *et al.* reported that the induction of hairy roots from *in vitro* leaf discs of *Duboisia* hybrid using *A. rhizogenes* strain A4 occurred in 80% of the infected leaf discs.[16] In this study, the leaf explants were prepared immediately after sterilization, which might have chemically damaged the leaf explants. This might be a reason for lower induction frequency, because approximately 25% of the explants died even after one day of co-culture with the lowest density (10^5) of bacteria (Fig. 2A). Nevertheless, hairy root induction from the leaf explants might be more convenient, because it does not require establishment of shoot culture. As Moyano *et al.*[5] reported, two types of roots, roots with typical hairy root mor-

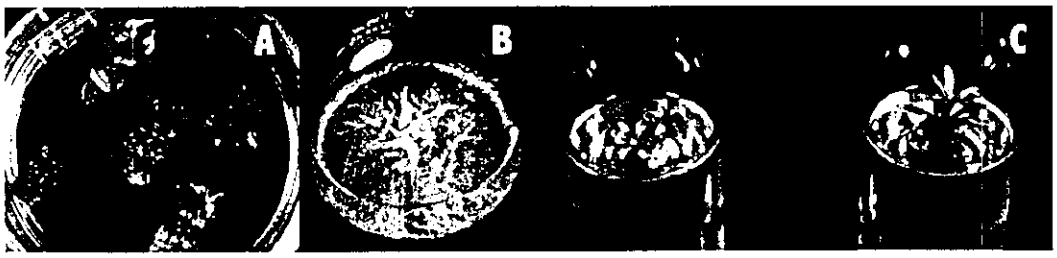

Fig. 1. Tissue Cultures of *Duboisia* Hybrid

A: Induction of hairy roots by co-culture method. Infected segments were cultured on 1/2 MS solid medium containing 500 mg/l Claforan® at 25 °C in the dark. B: The selected hairy root clone that demonstrated typical hairy root morphology. The hairy roots were cultured in MS liquid medium at 25 °C in the dark for 4 weeks. C: The regenerated shoots from the hairy (left) and adventitious (right) roots. The shoots were cultured on MS solid medium at 25 °C under 16 h light.

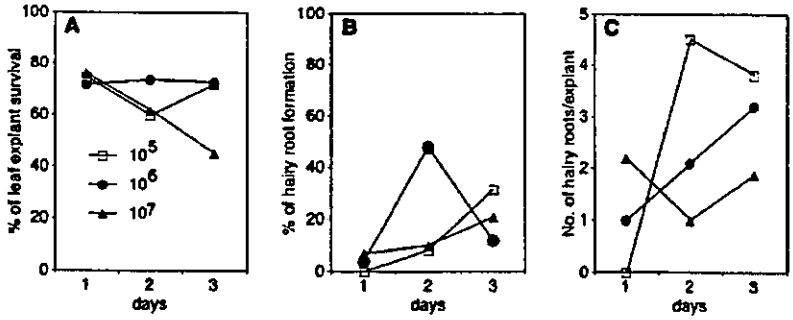

Fig. 2. Influence of Bacteria Density and Duration of Co-culture on Hairy Root Induction

The 15 leaf explants were used for each treatment. The leaf explants were co-cultured with *Agrobacterium rhizogenes* ATCC15834 (the bacteria density was set as 10^5, 10^6 or 10^7) for 1 to 3 d at 25 °C in the dark, and then transferred onto 1/2 MS solid medium containing 500 mg/l Claforan®. The leaf explant survival (A), frequency of hairy root formation (B) and number of hairy roots independently formed on the leaf explant (C) were observed after 3 weeks from co-culture.

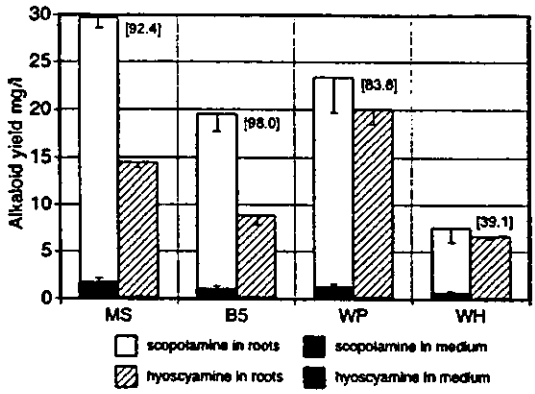

Fig. 3. Effects of Basal Media on the Growth and the Alkaloid Yield in the Hairy Roots of *Duboisia* Hybrid

The hairy roots (*ca.* 30 mg fresh weight) were inoculated into various basal liquid medium and cultured at 25 °C in the dark for 5 weeks. Bars represent standard deviation of the mean, $n=3$. Numbers in brackets show growth index on a dry weight basis. Growth index = final dry weight/initial dry weight.

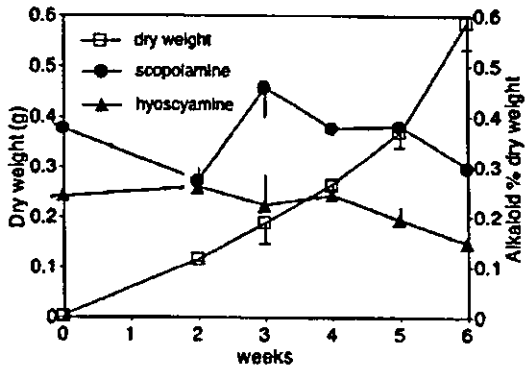

Fig. 4. Periodical Growth and Alkaloid Content in the Hairy Roots of *Duboisia* Hybrid in MS Liquid Medium

The hairy roots (*ca.* 30 mg fresh weight) were cultured in MS liquid medium in the dark at 25 °C. Bars represent standard deviation of the mean, $n=2$.

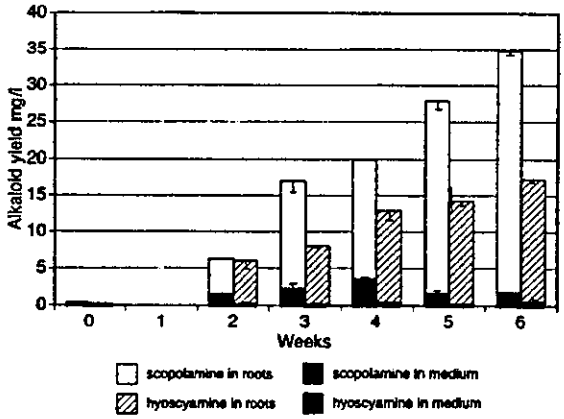

Fig. 5. Periodical Yields of Alkaloids in Hairy Root Cultures of *Duboisia* Hybrid

The culture conditions were the same as Fig. 4. Bars represent standard deviation of the mean, $n=2$.

phology (Fig. 1B) and callus-like roots, appeared. However, spontaneous shoot regeneration from *Duboisia* hairy roots previously reported[15,16] was not observed.

Growth and Tropane Alkaloid Production in Hairy Root Cultures The root clone with typical hairy root morphology growing fastest among the clones was selected and used for further experiments. The effects of basal media on the growth and alkaloid yield in the hairy roots are shown in Fig. 3. The best growth (growth index: 98.0) was observed in B5 medium, and the highest scopolamine content (0.38% dry weight) was obtained in MS medium. On the other hand, the highest hyoscyamine content (0.30% dry weight) was obtained in WP medium.

The best alkaloid productivity (30 mg/l scopolamine and 14 mg/l hyoscyamine) was obtained in MS medium after 5 weeks of culture. In our previous study on production of tropane alkaloids by adventitious root cultures of the same *Duboisia* hybrid, 13 mg/l scopolamine and 1.7 mg/l hyoscyamine were obtained in MS medium with 0.5 mg/l IAA after 5 weeks of culture.[12] The *Agrobacterium rhizogenes* transformation enhanced the tropane alkaloid productivity; over twice the amount of scopolamine and 8 times of hyoscyamine when compared with the adventitious roots.

Growth curve and the alkaloid contents of the hairy roots in MS medium are shown in Fig. 4. The hairy roots grew linearly toward the end of the culture at week 6, though the growth of adventitious roots with 0.5 mg/l IAA reached stationary state at week 3.[12] The contents of scopolamine (0.27 to 0.46% dry weight) and hyoscyamine (0.15 to 0.26% dry weight) were relatively constant during the whole culture period in contrast to the transient decrease of scopolamine and the transient increase of hyoscyamine contents in the early

stage of culture (week 1 to 3) observed in the adventitious roots.[12] The alkaloid yield in each culture period is shown in Fig. 5. Constant alkaloid content and linear increase of dry mass (Fig. 4) were reflected in the growth-proportional increase of tropane alkaloid yields (Fig. 5). At week 6, 35 mg/l scopolamine and 17 mg/l hyoscyamine were obtained.

Distributions of IAA and Scopolamine in Hairy and Adventitious Roots Since there were differences in the growth and alkaloid contents between hairy and adventitious roots, distributions of IAA and scopolamine along the root axis were determined by ELISA (Fig. 6). There were great differences in the distributions of scopolamine and IAA. Partial variation of contents both in scopolamine and IAA was obvious in the hairy roots, while it was not distinct in the adventitious roots. Inverse correlation of the contents was observed between scopolamine and IAA; increase of scopolamine and decrease of IAA were proportional to the distance from the root meristem in the hairy roots. In contrast the concentrations of scopolamine and IAA were less varied in the adventitious roots. Since all IAAs detected in the hairy roots were biosynthesized in the roots, relationship between IAA and scopolamine contents might appear clearly in the hairy roots. It is noteworthy that high content of IAA seems to be correlated with lower content of scopolamine in the hairy roots. It might reflect direct or indirect effect of IAA on accumulation or production of scopolamine, because the hairy roots were not supplied with any exogenous IAA. Increase of IAA level in the roots can decrease scopolamine levels. This is observed in the time course study of adventitious roots; a decrease of scopolamine content was detected at the early growth stage when exogenous IAA was supplied to their growth.[12]

To elucidate the factors for the growth differences, Deno *et al.* measured the amount of IAA present in the hairy and adventitious roots of *Duboisia myoporoides* by HPLC, and detected fairly high contents in the hairy roots during the culture (185, 159 and 103 pmol/g fresh weight IAA at day 7, 17 and 35, respectively) in contrast to the lower contents in the adventitious roots (81 and 93 pmol/g fresh weight IAA at day 13 and 30, respectively).[17] They concluded that the difference of the IAA contents was not large enough to explain the difference in the growth; 2-fold dry mass in hairy roots com-

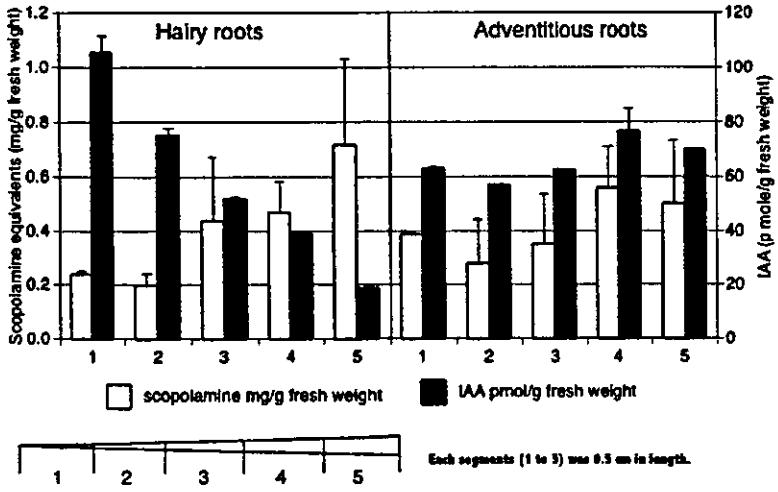

Fig. 6. Distributions of Scopolamine and IAA in the Hairy and Adventitious Roots Determined by ELISA

The hairy and adventitious roots were cultured in MS liquid medium at 25 °C in the dark for 4 weeks. Bars represent standard deviation of the mean, $n=4$.

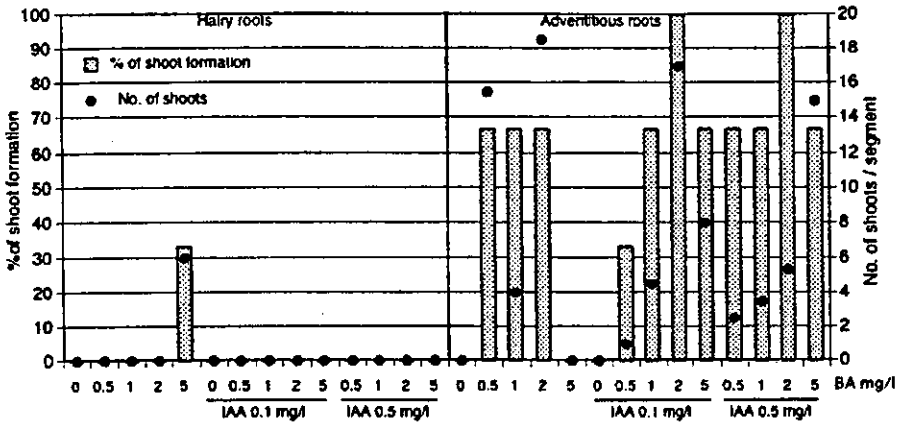

Fig. 7. Shoot Regeneration on the Hairy and Adventitious Root Segments of *Duboisia* Hybrid

The root segments grown in MS liquid media were cultured on 1/2 MS solid medium containing various concentrations and combinations of IAA and BA for 7 weeks at 25 °C under 16 h light. Three segments were used for each treatment.

pared to that in adventitious roots. However, an obviously lower content of scopolamine (0.15% dry weight) in hairy roots compared to that in adventitious roots (0.70% dry weight) was reported in their paper.[17] This is in accordance with our results for hairy roots; the second lowest content of scopolamine (0.24 mg/g fresh weight) and highest content of IAA (106 pmol/g fresh weight) in the root meristem.

Shoot Regeneration In most cases of shoot regeneration from hairy roots of *Duboisia*, spontaneous regeneration has been reported.[15,16] Establishment of a shoot regeneration system from desired clones is necessary for crop improvement. Therefore, shoot regeneration from hairy roots lacking spontaneous regeneration capability was investigated. In a preliminary experiment, shoot formation was examined both in the dark and under 16 h light. Frequency of shoot formation and number of shoots under light were much higher than in the dark. Therefore the adventitious and hairy root segments grown in the liquid medium were cultured with various concentrations and combinations of IAA and BA under 16 h light (Fig. 7). In adventitious roots, shoots regenerated on media containing BA (0.5 to 5 mg/l); 100% regeneration with 17 shoots per segment in medium with 0.1 mg/l IAA and 2 mg/l BA. On the other hand, shoot regeneration in hairy roots was only observed on the medium containing 5 mg/l BA at a rate of 33%. The hairy root segments which appeared to be more sensitive to IAA, formed vigorous callus on all the media containing IAA, and no shoot regeneration occurred. Callusing preceded shoot regeneration in all the cases, which was more prevalent in hairy roots. The shoots formed on the adventitious root segments grew well after transferring onto MS solid medium, whereas the shoots from the hairy roots formed callus at the basal end on MS solid medium and required cytokinin to maintain shoot morphology. From the results of IAA distribution (Fig. 6), IAA content in root meristems of hairy roots was 1.7 times higher than that of adventitious roots. Active callusing and enhanced sensitivity to exogenous supply of IAA might be attributed to this capability to synthesize IAA in hairy roots.

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Probing biosynthesis of plant polyketides with synthetic *N*-acetylcysteamine thioesters

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Abstract

Recombinant chalcone synthase (CHS) from *Scutellaria baicalensis* accepted cinnamoyl diketide-NAC and cinnamoyl-NAC as a substrate, and carried out sequential condensations with malonyl-CoA to produce 2′,4′,6′-trihydroxychalcone. Steady-state kinetic analysis revealed that the CHS accepted the diketide-NAC with less efficiency, while cinnamoyl-NAC primed the enzyme reaction almost as efficiently as cinnamoyl-CoA. On the other hand, it was for the first time demonstrated that the diketide-NAC was also a substrate for recombinant polyketide reductase (PKR) from *Glycyrrhiza echinata*, and converted to the corresponding β-ketohemithioester. Furthermore, by co-action of the CHS and the PKR, the NAC-thioesters were converted to 6′-deoxychalcone in the presence of NADPH and malonyl-CoA.

Keywords: Chalcone synthase; 6′-Deoxychalcone; NAC-thioester; Plant polyketide; Polyketide reductase

Chalcone synthase (CHS) (EC 2.3.1.74) is a pivotal enzyme in the biosynthesis of flavonoids as well as a wide range of biologically important natural products [1,2]. The type III polyketide synthase (PKS) utilizes free CoA thioesters as substrates without the involvement of acyl carrier protein. Thus, CHS catalyzes sequential condensation of 4-coumaroyl-CoA (**1a**) or cinnamoyl-CoA (**1b**) as a starter with three C_2 units from malonyl-CoA (**2**). After three rounds of the polyketide chain elongation reaction, Claisen-type cyclization of the enzyme-bound tetraketide intermediate leads to formation of 4,2′,4′,6′-tetrahydroxychalcone (naringenin chalcone) (**3a**) or 2′,4′,6′-trihydroxychalcone (pinocembrin chalcone) (**3b**), respectively (Fig. 1). Further, in enzyme reactions in vitro, triketide and tetraketide α-pyrones are also obtained as early released derailment by-products (Fig. 1) [3,4]. CHS is a homodimer of relatively modest-sized proteins of 40–45 kDa, and recent structure-based site-directed mutagenesis studies have revealed the catalytic mechanism of the chalcone forming reaction, which proceeds through starter CoA loading at the active site cysteine residue [5–16].

The promiscuity and functional diversity of the type III PKS enzymes are remarkable. In previous studies, we demonstrated that recombinant CHS from *Scutellaria baicalensis* (Labiatae) has unusually broad substrate specificities toward the non-physiological starter and extender substrate [17–22]. Thus, the CHS accepted a variety of aromatic and aliphatic CoA esters as a substrate, and efficiently yielded a series of chemically and structurally different unnatural polyketides. To further explore the catalytic potential of the enzyme, here we describe the enzymatic conversion of *N*-acetylcysteamine (NAC) thioester of cinnamic acid (cinnamoyl-NAC) (**6**) [23] and a NAC derivative corresponding to the diketide intermediate (cinnamoyl diketide-NAC) (**7**) [24] (Fig. 2). Although NAC-thioesters have been

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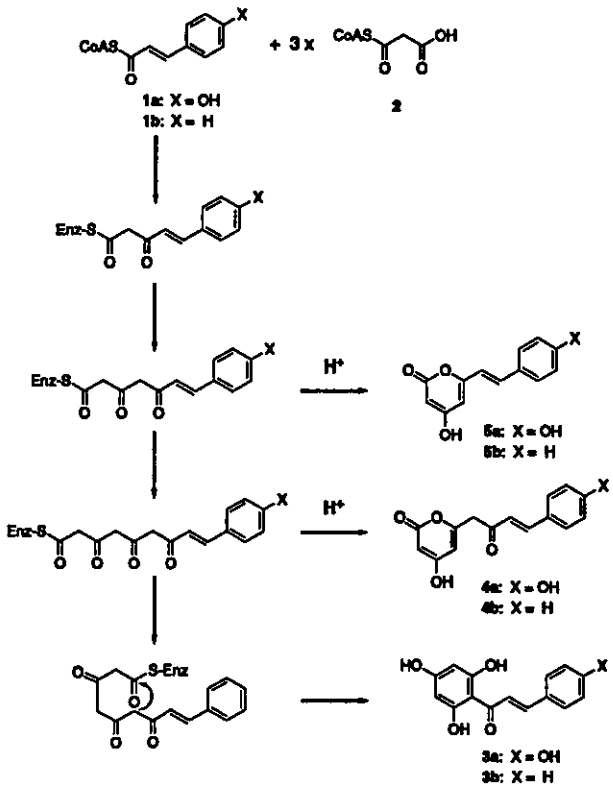

Fig. 1. Proposed mechanism for formation of chalcones from 4-coumaroyl-CoA (1a) and cinnamoyl-CoA (1b) by CHS.

successfully used to probe the catalytic function of polyketide synthases from plant and bacterial origin [25], this is the first report for the detailed analysis of enzyme kinetics as well as full characterization of the enzyme reaction products.

On the other hand, co-action of CHS and a NADPH-dependent polyketide reductase (PKR) leads to formation of a reduced chalcone (referred to as 6′-deoxychalcone) [11,26–31]. PKR is a monomeric protein of 34 kDa, and its cDNAs have been cloned, sequenced, and functionally expressed in *Escherichia coli* from several legume plants including *Glycine max* [28,29] and *Glycyrrhiza echinata* (Fabaceae) [30,31]. It has been reported that *G. max* PKR catalyzed transfer of the *pro-R* hydrogen of NADPH [27]. Although the buried nature of the CHS active site precludes PKR from accessing the CHS-enzyme-bound polyketide intermediate [2], PKR is thought to reduce a carbonyl group of the linear polyketide intermediate prior to formation of the aromatic ring system of the chalcone. To understand the mechanism of the 6′-deoxychalcone biosynthesis, here we investigated enzymatic conversion of the NAC-thioesters by recombinant *G. echinata* PKR. It was for the first time demonstrated that the NAC-thioesters were converted to 6′-deoxychalcone by the co-action of the CHS/PKR enzyme system.

Materials and methods

Materials. Cinnamoyl-NAC [(*E*)-phenylprop-2-enethioic acid *S*-[2-(acetylamino)ethyl]ester] (6) [23] and cinnamoyl diketide-NAC [(*E*)-3-oxo-5-phenylpent-4-enethioic acid *S*-[2-(acetylamino)ethyl]ester] (7) [24] were synthesized according to the published method. Spectroscopic data (NMR, UV, and MS) were completely identical with those of the published data. Cinnamoyl-CoA and 4-coumaroyl-CoA were chemically synthesized as described before [32]. [2-^{14}C]Malonyl-CoA (48 mCi/mmol) was purchased from Moravek Biochemicals (California). Recombinant *S. baicalensis* CHS [17,18] and recombinant *G. echinata* PKR [30,31] with an additional hexahistidine tag were expressed in *E. coli* and purified by Ni-chelate affinity chromatography as described before.

CHS enzyme reaction. The standard reaction mixture contained 54 μM of starter substrate (cinnamoyl-CoA, cinnamoyl-NAC, or diketide NAC), 108 μM of malonyl-CoA, and 5 μg of the purified enzyme in a final volume of 500 μL of 100 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA. Incubations were carried out at 30 °C for 20 min to overnight and stopped by adding 50 μL of 20% HCl. The products were then extracted with 1 mL of ethyl acetate (2×), concentrated by N_2 flow, and separated by HPLC as described below.

Steady-state kinetic parameters were determined by using [2-^{14}C]malonyl-CoA (1.8 mCi/mmol) as a substrate. The experiments were carried out in triplicate using five concentrations (10.8, 21.6, 32.4,

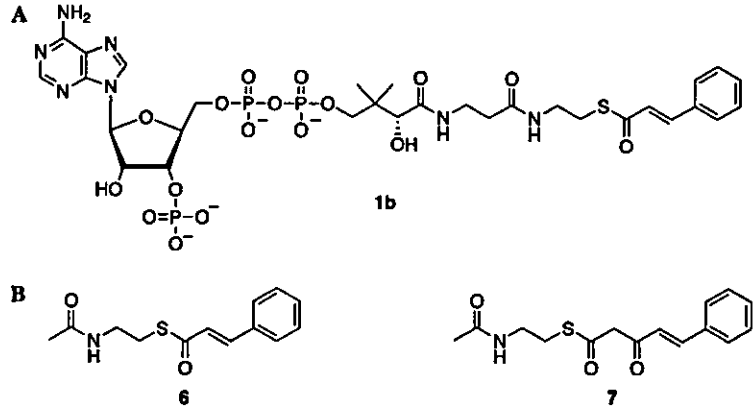

Fig. 2. Structures of (A) cinnamoyl-CoA (1b); (B) cinnamoyl-NAC (6) and cinnamoyl diketide-NAC (7).