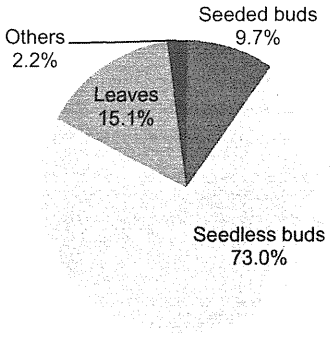

Fig. 4. Weight of marijuana in each class (total 286.6 kg).

On the other hand, CBN/THC can be an indicator for the freshness of marijuana [20]. Fig. 6 shows the relationship between CBN/THC and THC levels in marijuana samples. It was shown that the higher the CBN/THC was, the lower THC was, indicating long storage, possibly at ambient temperature or without cover from lights.

So marijuana samples of CBN/THC not exceeding or equal to 0.1 were regarded as 'fresh' and the data were chosen for statistics. Table 6 shows the results. The number of data was 424. The average THC and CBN levels in seedless buds were 11.2 and 0.7%, respectively, making sum of 11.9%. They were considerably higher than that of unselected samples.

Fig. 7 shows numbers of seeded buds in each class of THC levels, and Fig. 8 shows the numbers of seedless buds. The most frequent THC levels were 5–10% for seeded buds and 10–15% for seedless buds.

3.4.2. Regional characteristics

Table 7 shows the THC levels of marijuana from each area. No apparent difference was observed among them.

An irregular marijuana seizure was found in the Yokohama area. It was in tablet form, and its weight was 2.5 g and its size was 13 mm in diameter with a thickness of 9 mm. It contained 23.6% THC, 3.2% CBN, and no CBD. This was larger than the usual ecstasy tablets and was partly shaved, indicating divisional use for smoking, not for oral administration.

3.4.3. CBD levels

CBD was found in 86 samples out of 1115. The highest level was 11.5% in a seedless buds sample. In most samples, the CBD content was below 2%. The World Drug Report 2006 mentioned the following: 'CBD is believed to 'moderate' the effects of THC, promoting relaxation and possibly even having an antipsychotic effect.' 'Where possible, it would be advisable to track both THC and CBD levels in future evaluations of 'potency' [12].' The present study shows that the majority of Japanese illicit cannabis products contain little or no CBD.

Supposed CBC was detected in 977 samples out of 1115. Its concentrations in marijuana were up to 1.7% if the response factor of CBC/CBN was assumed to be 1. In 52 samples, neither CBD nor CBC was detected.

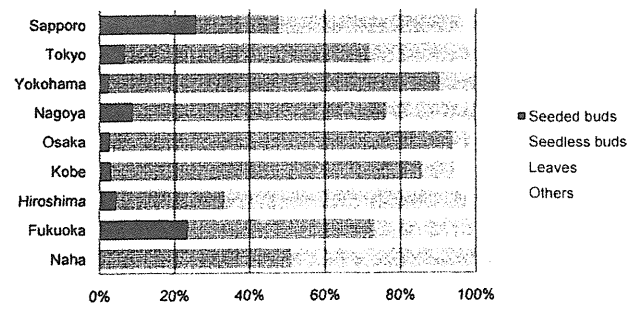

Fig. 5. Ratio of marijuana class in each area of Japan. Data from Sendai and Takamatsu are not included because the numbers were too small.

Table 5
Cannabinoids levels in *all* marijuana samples tested.

	Number of samples	Average (range) (%)		
		THC	CBN	CBD
Seeded buds	100	3.8 (ND-16.4)	1.1 (ND-4.7)	0.2 (ND-4.3)
Seedless buds	758	8.3 (ND-22.6)	1.3 (ND-6.4)	0.1 (ND-11.5)
Leaves	254	1.8 (ND-14.2)	0.5 (ND-3.4)	0.2 (ND-6.2)
Powder and tablet	3	8.9 (0.3–23.6)	1.2 (0.1–3.2)	0.1 (ND-0.3)
Total	1115			

ND: not detected (<0.1%).

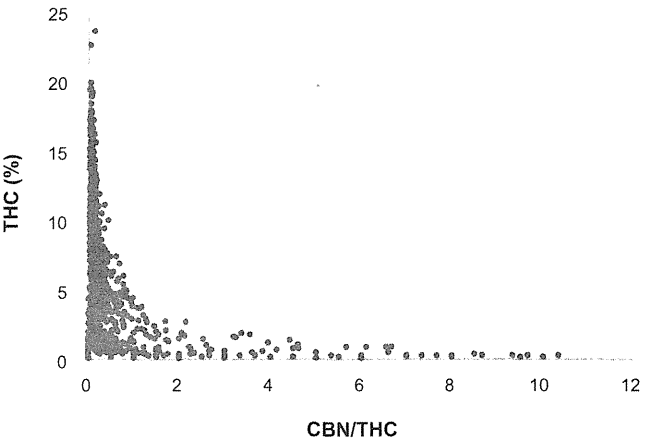

Fig. 6. Relationship between CBN/THC and THC levels in marijuana.

3.5. Assessment for the results

Several researchers have reported increased THC contents in marijuana. Mehmedic et al. reported that 1093 Sinsemilla samples

Table 6
Cannabinoids levels in *fresh*[a] marijuana samples tested.

	Number of samples	Average (range) (%)		
		THC	CBN	CBD
Seeded buds	28	7.1 (0.8–16.4)	0.4 (ND-1.3)	0.3 (ND-4.3)
Seedless buds	335	11.2 (0.2–22.6)	0.7 (ND-1.6)	0.1 (ND-11.5)
Leaves	60	3.3 (0.1–14.2)	0.2 (ND-1.3)	0.1 (ND-2.1)
Powder	1	2.7	0.1	0.3
Total	424			

ND: not detected (<0.1%).
[a] Samples with CBN/THC below or equal to 0.1.

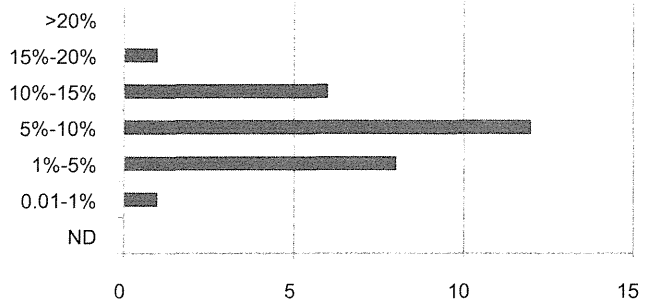

Fig. 7. Sample numbers of fresh *seeded* buds classified by THC levels.

in the USA in 2008 contained 11.5% THC on average [1]. Potter et al. reported that 247 Sinsemilla samples in the UK in 2004/5 contained a median of 13.98% THC [2]. The home office of the UK reported in 2008 that the average level of sinsemilla was 16.2% [3]. Pijlman et al. reported that 62 Nederwiet (Dutch domestic cannabis) samples from the Netherlands in 2004 contained an average of 20.4% and a median of 21.5% THC [4]. A German survey reported the average THC level in 9250 seized marijuana specimens to be 8% in 2009 [5]. Licata et al. reported that 98 Marijuana samples from Italy in 2004 contained an average of 15% THC [6]. Knight mentioned that an investigation of 43 cannabis seizures in New Zealand showed an average of 10.9% THC, and the experimental grow of the seized cannabis resulted in a range of 4.3–25.2% THC in sinsemilla [7].

We found an average of 11.2% THC in seedless buds. The highest was 22.6%. These levels are comparable to the 'high potency cannabis' in the studies mentioned above.

In Japan, the THC contents of licit or wild cannabis were surveyed by the Ministry of Health and Welfare in 1970 [16]. At that time, majority of abused cannabis was imported one, but many cases were also reported with domestic cannabis. Abusers stole them at farmers' fields or picked them in suburbs or remote area [16]. Thus the data represents certain part of cannabis being abused in that time and can be used for comparison, even though the information is limited. According to the report, THC levels were highest in products from Tochigi (a prefecture near Tokyo), 4%, followed by the wild cannabis in Hokkaido, 3.4%. The lowest was a product of Kyushu, 0.1%. The average THC in Japanese cannabis was around 1%. The present study showed greater THC contents in recent marijuana than was observed in the 1970 research, even in the leaves or seeded buds.

The origins of seized marijuana in the present survey are not clear. However, in Japan, import cases of cannabis are decreasing and cultivation cases are increasing dramatically in the last decade. Statistics for 2010 show the number of cultivation cases (196) is 8 times as high as that of import cases (25) [14]. That fact indicates that majority of Japanese illicit cannabis is domestic one now.

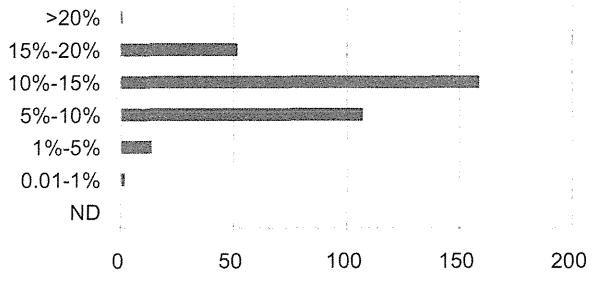

Fig. 8. Sample numbers of fresh *seedless* buds classified by THC levels.

Table 7
THC levels in fresh seedless buds in each area of Japan.

City	Number	THC levels (%)		
		Average	SD	Max
Sapporo	22	10.2	4.2	19.9
Sendai	6	12.7	2.1	16.2
Tokyo	86	11.7	3.7	19.5
Yokohama	39	12.7	3.9	19.2
Nagoya	35	11.9	3.2	22.6
Osaka	72	10.3	3.2	17.4
Kobe	37	9.6	3.2	17.9
Hiroshima	14	10.9	5.4	19.4
Fukuoka	22	11.1	3.2	17.4
Naha	2	13.3	4.0	17.2
Total	335			

With regard to the 'difference' in the THC levels of each marijuana product, we can see the distribution in Figs. 7 and 8. The most frequently encountered THC levels were between 10 and 15% in seedless buds. When these levels were considered as standard, almost all cannabis products contained less than two times these levels. Therefore, the possibility of an unexpected overdose of THC may not be large. However considering that some cannabis products with less than 5% THC were found, unexpected overdose cannot be completely avoided.

The present study is the first survey on Japanese illicit cannabis. It showed the distribution of 'high potency cannabis' in Japan as has been shown in many other countries, thus showing the necessity of continuous monitoring. Since the post-trial cannabis samples showed decreased THC content, cannabis should be surveyed immediately after seizure.

4. Conclusions

The features and cannabinoids contents of Japanese illicit cannabis products were investigated. Seedless buds, commonly known as 'sinsemilla', represent the majority of marijuana seizures in Japan. The average THC level in relatively fresh seedless buds was 11.2%. The highest THC level observed was 22.6%. These facts indicate the distribution of 'high potency cannabis' in Japan. The present paper is the first report for cannabis potency in Asian area, covering almost whole seizures in one nation, using standardized methods for quantification.

Acknowledgements

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絶え間なく出現する違法ドラッグを化学の力で識別する

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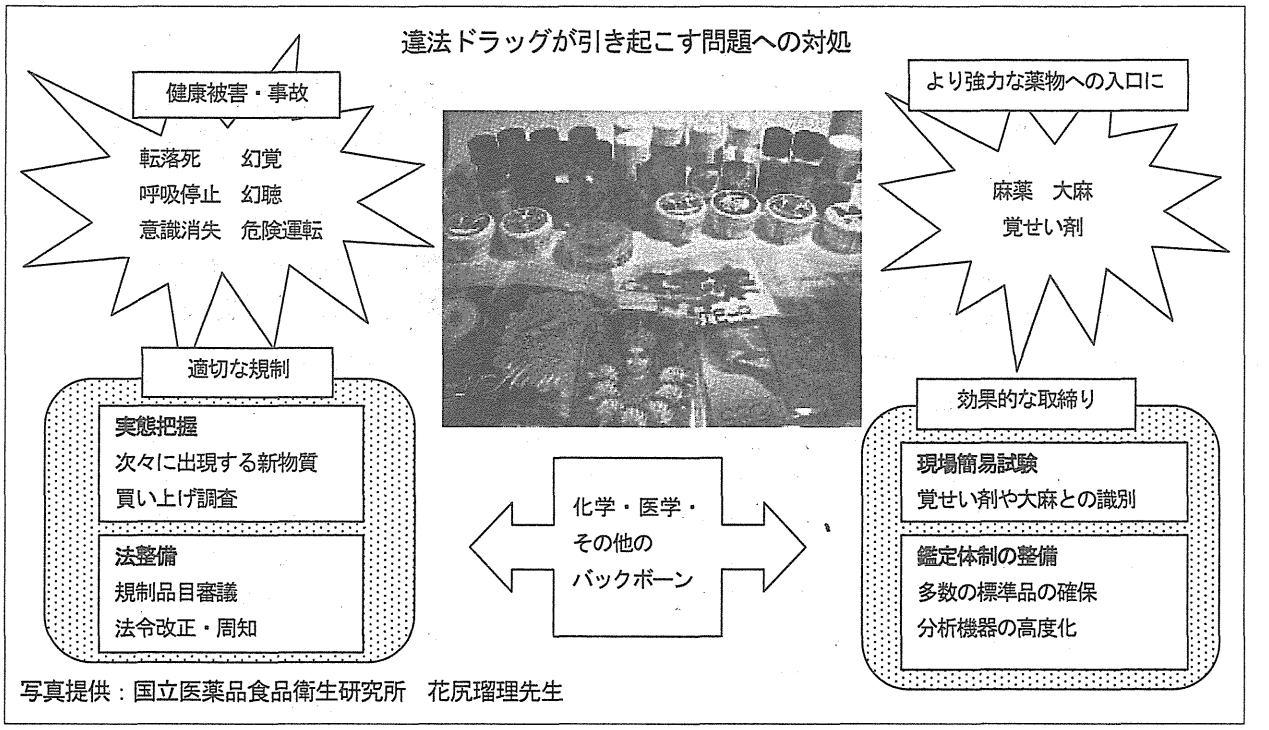

最近「合法（脱法）ハーブ」「合法（脱法）ドラッグ」といった言葉がニュースによく登場する。これらはアダルトショップやネットショップで販売される植物・錠剤・粉末・液体状の薬物で，精神作用を期待して摂取されるが，意識消失などの思わぬ副反応のために摂取者が救急搬送されたり交通事故を起こしたりした事例が報道されている。

厚生労働省は「合法ドラッグ」や「脱法ドラッグ」の語を用いず，これらを「違法ドラッグ」と呼んでいる。というのは，人に摂取させる目的でこれらを販売することは薬事法に違反するからだ。

違法ドラッグは化学的にはどのような物質なのだろうか。広く出回っているものとしては，合成カンナビノイド，フェネチルアミン類，トリプタミン類，ピペラジン類，亜硝酸エステル類，様々な成分を含む植物等[1,2]が挙げられる（図1）。これらは薬事法の規制を逃れるために「芳香剤」「ビデオクリーナー」等の名目で販売されるものが多く，十分な取締りができていなかった。そこで2007年4月から指定薬物制度が導入された。

指定薬物は，化学構造などから中枢神経系の作用と保健衛生上の危害が予想されるもので，厚生労働大臣が薬事・食品衛生審議会の意見を聴いて指定する。麻薬や覚せい剤ほどではないが，未規制のドラッグよりも厳しく取締りを受ける（表1）。

乱用薬物の取締りに欠かせないのは化合物の正確な識別だ。ほんの少し化学構造が違う一群の物質の中で，あるものは麻薬，あるものは指定薬物，あるものは未規制物質に該当する例は多い（図2）。鑑定を担当する機関では，様々な分析機器を使ってこれらの薬物を識別する体制を整えている[3]（図3）。

市販される違法ドラッグ製品から麻薬の成分が検出された例もある。危険な薬物乱用を防ぐため，化学の力が役立っている。

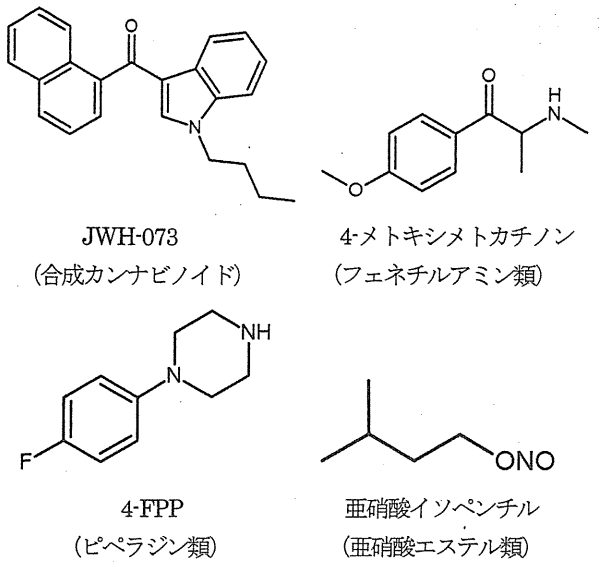

図1　違法ドラッグの化学構造式：主要な化合物群から発見例の多いものをそれぞれ1物質ずつ挙げた。実際には，側鎖等をわずかに変えたものが非常に多数出回っている。ただし合成カンナビノイドは全く違う構造の化合物群が複数存在する。「脱法ハーブ」は，植物片に合成カンナビノイドが加えられたものである場合が多い。

表1　違法ドラッグと麻薬・覚せい剤・大麻との違い。

	麻薬・覚せい剤・大麻*	違法ドラッグ	
		指定薬物	未規制の薬物
法律	麻薬及び向精神薬取締法等	薬事法	薬事法
該当要件	法令に物質名記載	法令に物質名記載	人体への摂取を目的に販売等した場合
品目数(2012年7月現在)	157	78	無数
販売	×	×	×
所持	×	未規制	未規制
使用	× (大麻は未規制)	未規制	未規制
指定要件	多くのデータや審議必要	比較的少ないデータで可能	—

*麻薬原料植物や向精神薬等も規制があるがこの表では除外。

表1は3つのカテゴリーの薬物がどのように規制されているかを示す。左側のものほど厳しく規制されている。指定薬物の販売は医療用途など特定の場合にのみ認められる。要件を満たした未規制物質は指定薬物に加えられるが，少し構造を変えた新たな薬物が出回り，いたちごっことなっている。

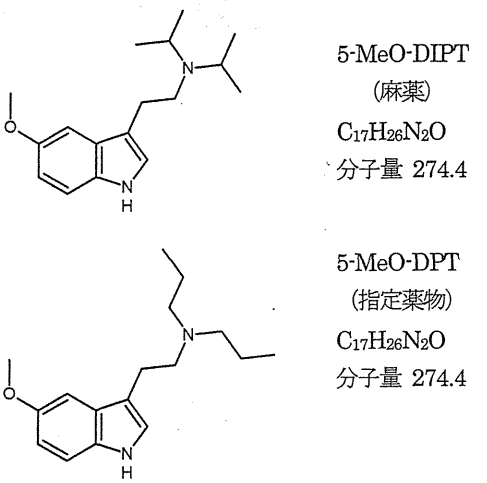

図2　類似した構造の麻薬と指定薬物：いずれもトリプタミン類で，側鎖が異なる異性体。

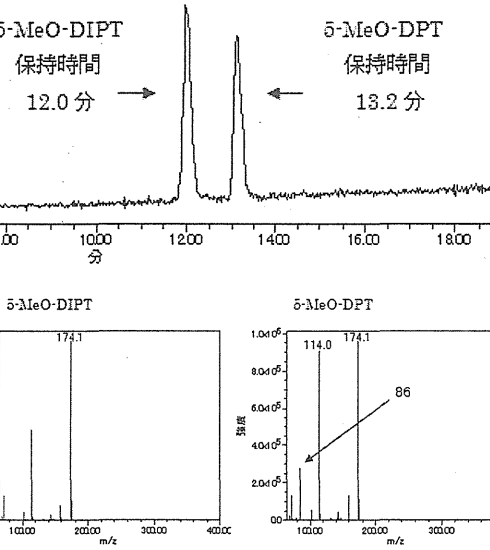

図3　液体クロマトグラフィー質量分析による5-MeO-DIPTと5-MeO-DPTの識別：上の図はクロマトグラム，下の図は質量スペクトル。これに対してガスクロマトグラフィー質量分析で分析した場合は，保持時間，スペクトルとも違いが少なく，識別が難しい。

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Article

Genetic and Phenotypic Analyses of a *Papaver somniferum* T-DNA Insertional Mutant with Altered Alkaloid Composition

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Abstract: The *in vitro* shoot culture of a T-DNA insertional mutant of *Papaver somniferum* L. established by the infection of *Agrobacterium rhizogenes* MAFF03-01724 accumulated thebaine instead of morphine as a major opium alkaloid. To develop a non-narcotic opium poppy and to gain insight into its genetic background, we have transplanted this mutant to soil, and analyzed its alkaloid content along with the manner of inheritance of T-DNA insertion loci among its selfed progenies. In the transplanted T_0 primary mutant, the opium (latex) was found to be rich in thebaine (16.3% of dried opium) by HPLC analysis. The analyses on T-DNA insertion loci by inverse PCR, adaptor-ligation PCR, and quantitative real-time PCR revealed that as many as 18 copies of T-DNAs were integrated into a poppy genome in a highly complicated manner. The number of copies of T-DNAs was decreased to seven in the selected T_3 progenies, in which the average thebaine content was 2.4-fold that of the wild type plant. This may indicate that the high thebaine phenotype was increasingly stabilized as the number of T-DNA copies was decreased. In addition, by reverse transcription PCR analysis on selected morphine biosynthetic genes, the expression of codeine 6-*O*-demethylase was clearly shown to be diminished in the T_0 *in vitro* shoot culture, which can be considered as one of the key factors of altered alkaloid composition.

Keywords: *Papaver somniferum* L.; opium alkaloid; *Agrobacterium rhizogenes*; T-DNA insertional mutant

1. Introduction

Many attempts have been made to use breeding or molecular biological methods to modify the ability to produce secondary metabolites in medicinal plants. Among the challenges being addressed, manipulations of the morphine biosynthesis in the opium poppy (*Papaver somniferum* L.), particularly the conversion of narcotic morphine to codeine, which is of high importance as an antitussive and a synthetic source of dihydrocodeine, or to thebaine, which is also an important starting material for the semi-synthesis of the analgesic oxycodone, will contribute to the control of narcotics, and to the supply of useful alkaloids for the production of pharmaceuticals.

The gradual elucidation of enzymology of the alkaloid biosynthesis in *P. somniferum* led to genetical engineering of alkaloid biosynthetic pathway using native genes. The first report was on the introduction of a gene encoding berberine bridge enzyme (BBE) to *P. somniferum* in antisense orientation [1]. To date, several reports on metabolic engineering of *P. somniferum* have appeared, such as RNAi-mediated gene silencing of codeinone reductase (COR) [2], overexpression of COR [3], overexpression and antisense co-suppression of (*S*)-*N*-methylcoclaurine-3'-hydroxylase (CYP80B3) [4], overexpression and RNAi-mediated gene silencing of salutaridinol-7-*O*-acetyltransferase (SalAT) [5], and RNAi-mediated gene silencing of SalAT [6]. Mutant poppy *top1* [7] which accumulates thebaine and oripavine as major alkaloids instead of morphine was also established by the treatment of mutagen (ethyl methanesulphonate) and screening of progeny plants.

The T-DNA insertional mutant clone of *P. somniferum* PsM1-2, which we developed by the infection of the *Agrobacterium rhizogenes* strain MAFF03-01724, regenerated shoots from embryogenic callus that lacked the ability to produce morphine. Codeine was detected as a major alkaloid in this *in vitro* shoot culture [8]. By the improvement of the alkaloid analysis and proceeding studies on this mutant, thebaine (*ca.* 55 μg/g dry weight) and codeine (*ca.* 20 μg/g dry weight) were found to be the major opium alkaloids in the *in vitro* regenerated shoots [9]. The information provided from this mutant, which shows an altered alkaloid composition, might make an important contribution to the further modification of alkaloid production in *P. somniferum*, and therefore we carried out genetic and phenotypic analyses on this mutant.

Recently, long unidentified enzymes involved in the two demethylation steps in the conversion of thebaine to morphine were successfully identified as non-heme dioxygenases [10]. These two enzymes, namely, thebaine 6-*O*-demethylase (T6ODM) and codeine *O*-demethylase (CODM), represent the first known 2-oxoglutarate/Fe(II)-dependent dioxygenases that catalyze *O*-demethylation. The altered alkaloid composition in the PsM1-2 mutant may be due to the genetic mutation in the conversion steps from thebaine to morphine. In the present study, an expression analysis of these two enzymes together with selected genes involved in the morphine biosynthesis was carried out to reveal the molecular mechanism of the mutation.

2. Results and Discussion

2.1. Morphological Characteristics of the PsM1-2 Mutants

The days to flowering, number of petals, appearance of split on the boundary of the petal, and height of the aerial part at the seed-filling stage of soil-cultivated T_0 mutant and selfed progenies are summarized in Table 1. The T_0 primary mutant showed delay of flowering and dwarfness. In addition, a deep split was observed on the boundary of the petal (Figure 1). Delay of flowering was consistantly observed in the progenies. The number of petals, which was not altered at the T_0, varied in the T_1, T_2 and T_3 progenies. A deep split at the boundary of the petal was observed in 45% of T_1 plants, 33% to 83% of T_2 plants, and 8.3% and 10% of T_3 plants.

Table 1. Summary of the morphological characteristics of PsM1-2 T_0 mutant, selfed progenies, and WT plant.

Progenies	Lines	Number of Plants	Days to Flowering (Mean ± SD) (days)	Number of Petals: Percentage (%)	Split on Petal Boundary (%)	Plant Height (Aerial Part) (Mean ± SD) (cm)
T_0	WT	1	47 *[1]	4: 100	0	60.0
	T_0	1	71 *[1]	4: 100	100	38.0
T_1	WT	6	53.5 ± 4.8	4: 100	0	42.4 ± 5.8
	T_1	60	100.6 ± 14.6 [####]	3: 1.7, 4: 41.7, 5: 35.0, 6: 16.7, 7: 3.3, 8: 1.7	45.0	52.1 ± 8.5 [##]
T_2	WT	12	53.3 ± 4.0	3: 25.0, 4: 75.0	8.3	36.0 ± 7.6
	#1-27(HT)	15	90.8 ± 12.6 [####]	5: 60.0, 6: 33.3, 10: 6.7	60.0	44.7 ± 5.4 [##]
	#2-17(HT)	6	79.8 ± 2.5 [####]	5: 50.0, 6: 33.3, 8: 16.7	83.3	45.1 ± 3.4 [#]
	#2-1(LT)	12	83.3 ± 6.8 [####]	5: 66.7, 6: 16.7, 7: 8.3, 8: 8.3	33.3	35.6 ± 7.8
	#2-6(LT)	10	76.4 ± 3.6 [####]	5: 10.0, 6: 40.0, 7: 30.0, 8: 10.0, 12: 10.0	80.0	39.4 ± 3.1
T_3	WT	6	109.4 ± 0.9 *[2]	4: 100	0	80.3 ± 5.8
	#1-27(HT) L#2	10	129.2 ± 11.9 *[3,###]	4: 40.0, 5: 50.0, 6: 10.0	10.0	45.8 ± 7.9 [####]
	#2-17(HT) #2-1	12	131.1 ± 7.3 *[4,####]	3: 8.3, 4: 75.0, 5: 16.7	8.3	47.0 ± 13.4 [####]

*[1]: Days after transplanting; *[2]: n = 5; *[3]: n = 9; *[4]: n = 11; [#] $p < 0.05$; [##] $p < 0.01$; [###] $p < 0.005$; and [####] $p < 0.001$ *vs.* WT.

2.2. Alkaloid Composition in the PsM1-2 Mutants

The soil-cultivated PsM1-2 T_0 primary mutant accumulated 16.3% (% dry weight) of thebaine as a major opium alkaloid in the latex, which was not detected in the WT (Figure 2; Table 2). The

morphine content in the mutant was 1.3%, which was *ca.* one tenth of that in the WT, and the codeine content was 4.2% in the mutant, *vs.* 1.3% in the WT.

Figure 1. Appearances of the PsM1-2 T_0 primary mutant and WT *P. somniferum* soil-cultivated in the phytotron. **(A)** WT, **(B)** PsM1-2 T_0. Upper left: flower; right: grown plant; bottom left: petals with deep splits (PsM1-2 T_0 only).

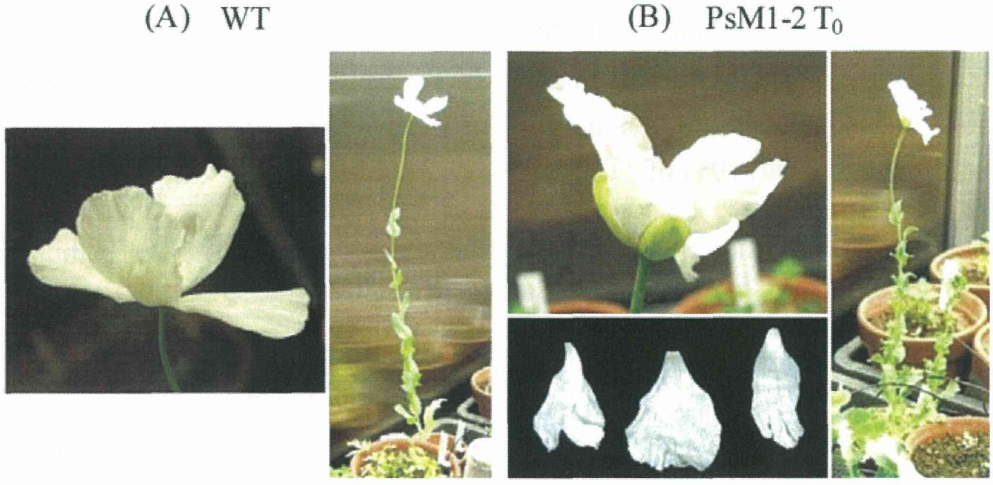
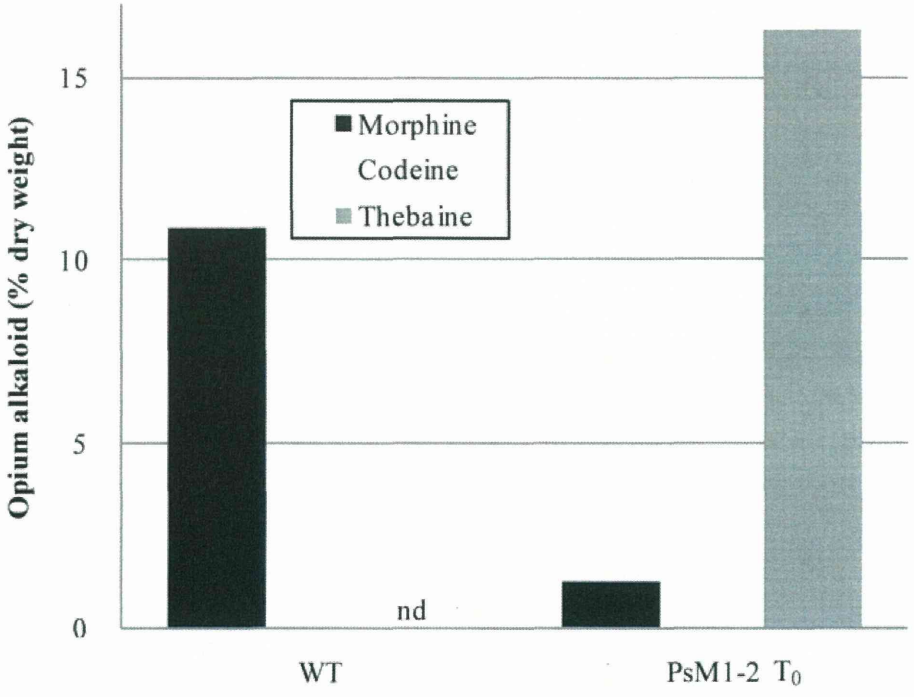


Figure 2. Alkaloid content in the latex from the soil-cultivated WT and PsM1-2 T_0 mutant. nd: Not detected.



The alkaloid compositions in the dried opium of selected progenies are summarized in Table 2, and the morphine and thebaine contents of the T_1, T_2 and T_3 plants are plotted on a scatter diagram (Figure 3). The HPLC chromatograms of the representative lines of the T_1 plants, WT plant, and authentic standards are shown in the Supplementary Figure 1.

Table 2. Opium alkaloid contents in PsM1-2 T_0 mutant and selfed progenies.

Progenies	Lines	Number of plants	Morphine	Codeine	Thebaine	Papaverine	Noscapine
T_0	WT	1	10.9	1.3	nd *	2.0	9.2
	T_0	1	1.3	4.2	16.3	2.3	10.2
T_1	WT	6	11.2 ± 4.0	2.6 ± 2.1	0.3 ± 0.2	2.4 ± 0.7	11.6 ± 4.3
	T_1	60	6.3 ± 4.6 $^{\#}$	3.8 ± 1.5	11.1 ± 6.1 $^{\#\#\#}$	1.6 ± 0.5	7.9 ± 2.1 $^{\#\#\#}$
Selected lines (T_1)	#1-27(HT)	-	4.3	5.1	23.1	2.3	7.2
	#2-17(HT)	-	5.5	3.7	24.4	1.9	6.8
	#2-1(LT)	-	23.0	1.3	0.3	1.8	8.4
	#2-6(LT)	-	13.6	2.1	1.0	1.5	6.9
T_2	WT	11	18.4 ± 3.3	1.5 ± 0.9	0.4 ± 0.2	2.7 ± 1.0	18.4 ± 4.3
	#1-27(HT)	15	7.0 ± 4.1 $^{\#\#\#}$	5.8 ± 1.6 $^{\#\#\#}$	19.1 ± 7.3 $^{\#\#\#}$	2.2 ± 0.4	9.6 ± 2.4 $^{\#\#\#}$
	#2-17(HT)	6	9.8 ± 8.1 $^{\#}$	6.0 ± 0.5 $^{\#\#\#}$	14.5 ± 6.1 $^{\#\#\#}$	3.0 ± 0.4	9.2 ± 1.7 $^{\#\#\#}$
	#2-1(LT)	12	7.6 ± 3.3 $^{\#\#\#}$	5.8 ± 1.1 $^{\#\#\#}$	15.9 ± 7.2 $^{\#\#\#}$	2.8 ± 0.8	8.0 ± 2.0 $^{\#\#\#}$
	#2-6(LT)	10	7.6 ± 6.8 $^{\#\#\#}$	4.6 ± 1.3 $^{\#\#\#}$	13.4 ± 6.7 $^{\#\#\#}$	2.7 ± 0.5	11.7 ± 2.7 $^{\#\#\#}$
Selected lines (T_2)	#1-27(HT) L#2	-	4.9	6.1	29.6	2.4	9.4
	#2-17(HT) #2-1	-	3.7	6.5	20.0	3.1	10.4
	#2-1(LT) #2-4	-	5.3	4.3	29.4	3.1	9.1
	#2-6(LT) #2-2	-	3.1	4.9	21.1	2.7	13.2
T_3	WT	6	11.1 ± 4.1	1.5 ± 0.5	3.3 ± 2.1	1.9 ± 0.5	4.2 ± 1.6
	#1-27(HT) L#2	10	2.5 ± 0.6 $^{\#\#\#}$	4.3 ± 0.4 $^{\#\#\#}$	7.7 ± 1.9 $^{\#\#}$	1.2 ± 0.1 $^{\#\#\#}$	5.1 ± 0.7
	#2-17(HT) #2-1	12	1.8 ± 0.5 $^{\#\#\#}$	2.9 ± 0.5 $^{\#\#\#}$	8.1 ± 2.3 $^{\#\#\#}$	1.1 ± 0.2 $^{\#\#\#}$	4.1 ± 0.8

Mean value of the alkaloid content (% dry weight) with standard deviation (mean ± SD) for each line and the alkaloid content of selected lines are summarized. nd *: Not detected; $^{\#}$ $p < 0.05$; $^{\#\#}$ $p < 0.005$; and $^{\#\#\#}$ $p < 0.001$ *vs.* WT.

The thebaine content in T_1 plants varied widely, from 0.3% to 26.5%. From these plants, two high thebaine lines, #1-27(HT) (thebaine content: 23.1%) and #2-17(HT) (24.4%), and two low thebaine lines, #2-1(LT) (0.3%) and #2-6(LT) (1.0%), were selected and subjected to analysis of the T_2 progeny. Interestingly, most of the progeny plants from both the HT and LT lines showed the high thebaine phenotype. From the T_2 lines, two lines, #1-27(HT)L#2 (thebaine content: 29.6%) and #2-17(HT)#2-1 (20.0%), were selected for the analysis of T_3 progeny.

The thebaine content in T_3 plants ranged from 4.2% to 10.0% in #1-27(HT)L#2 and from 3.7% to 10.9% in #2-17(HT)#2-1. The average thebaine content in T_3 plants (two lines combined) was 2.4-fold of that in the WT; in contrast, the average morphine content decreased to *ca.* one fifth of that in the WT (Figure 4).

Figure 3. Alkaloid content in the latex from the soil-cultivated WT and PsM1-2 T_0 mutant. nd: Not detected. Scatter diagram of the morphine (x-axis) and thebaine (y-axis) contents in (**A**) PsM1-2 T_1 plants (n = 60) and WT plants (n = 6), (**B**) four lines of PsM1-2 T_2 plants and WT plants, and (**C**) two lines of PsM1-2 T_3 plants and WT plants.

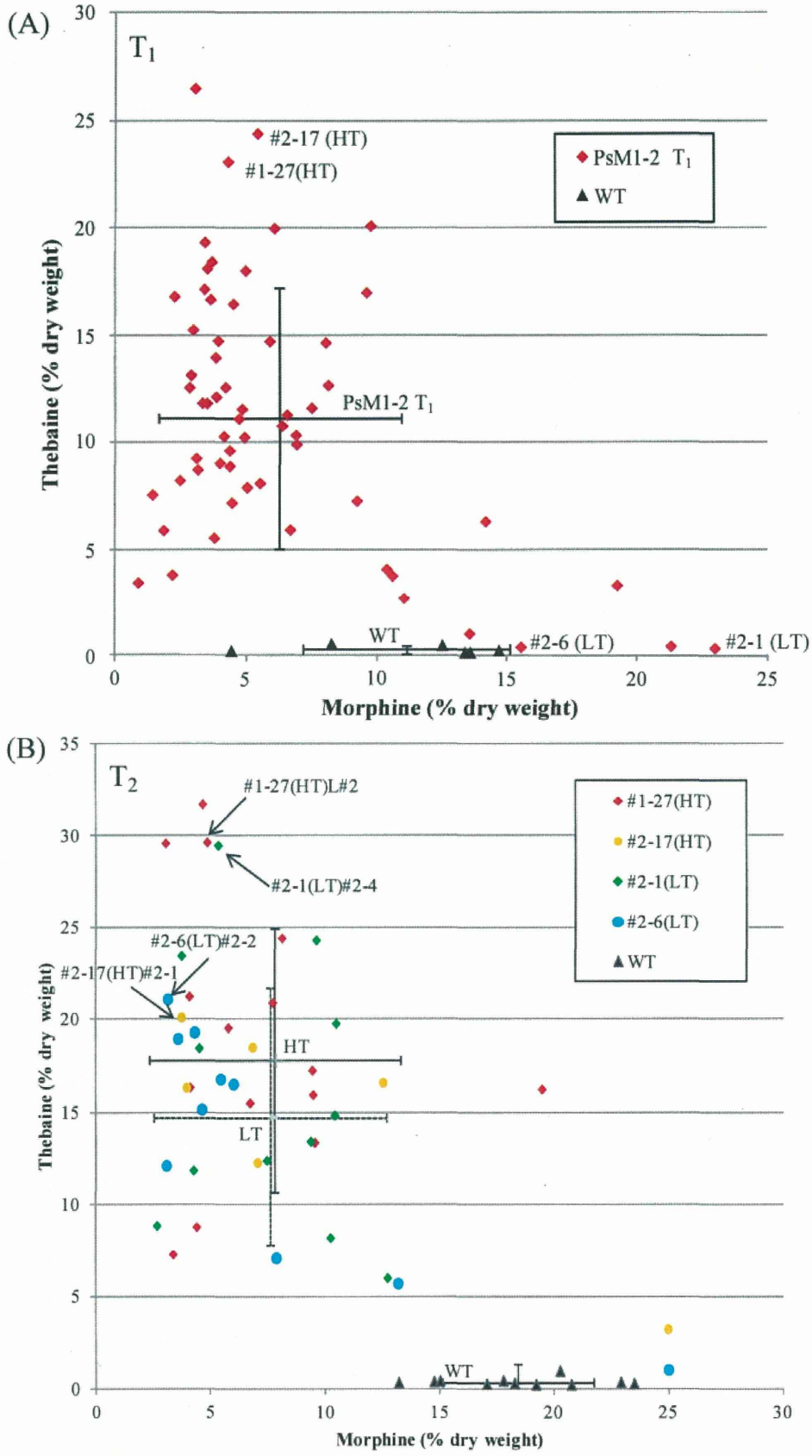
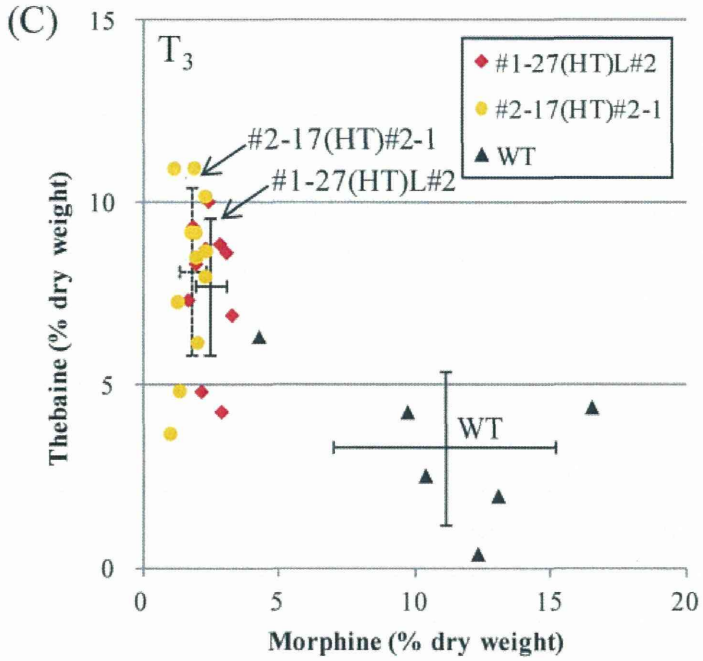
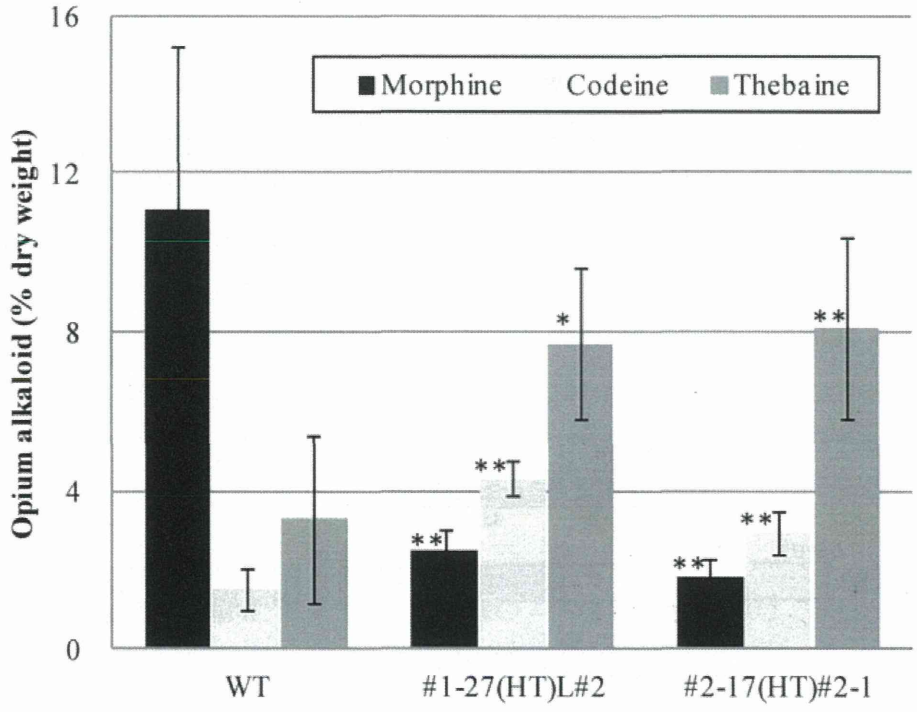


Figure 3. *Cont.*



Crossed bars indicate the mean value (crossed point) and standard deviations for morphine (horizontal bar) and thebaine (vertical bar) contents in (**A**) PsM1-2 T_1 plants and WT plants, (**B**) HT (#1-27 and #2-17 were combined) and LT (#2-1 and #2-6 were combined, dotted) lines, and (**C**) WT, #1-27(HT)L#2, and #2-17(HT)#2-1 (dotted) plant lines. (**A**) Lines #1-27(high thebaine, HT), #2-17(HT), #2-1(low thebaine, LT), and #2-6(LT) were used for analyses on T_2 progeny. (**B**) Lines #1-27(HT)L#2 and #2-17(HT)#2-1 were used for analyses on T_3 progeny.

Figure 4. Morphine, codeine, and thebaine contents in T_3 progeny. Mean value of six (WT), 10 [#1-27(HT)L#2], and 12 [#2-17(HT)#2-1] plants. Bars indicate standard deviation. * $p < 0.005$ and ** $p < 0.001$ *vs.* WT.



2.3. T-DNA Insertion Loci Analysis by IPCR and AL-PCR

The genomic DNA regions adjacent to the inserted T-DNA borders were analyzed by the IPCR and AL-PCR methods. The obtained DNA fragments are summarized in Supplementary Table 1 and Figure 5 along with the PCR methods, the combination of template circular or adaptor-ligated genome DNA libraries, and the primer sets. Sequence analysis of the amplified products revealed that the fragments were classified into three types, (A) T-DNAs connected with *P. somniferum* genome DNA, (B) T-DNAs connected in tandem, and (C) T-DNAs connected with T-DNA internal fragments, as shown in Figure 5.

Type (A) includes four types of genome DNA fragments adjacent to T-DNA LB, and six types of genome DNA fragments adjacent to T-DNA RB. Of these fragments, LB1g and RB2g, LB3g and RB6g were confirmed to be both ends of single genomic loci, by PCR over the LB and RB genomic regions. Although the tally of paired border for other fragments were not found, at least eight independent T-DNA integrated sites, namely RB1, RB2 (LB1-RB2), RB3, RB4, RB5, LB2, LB3 (LB3–RB6), and LB4 were estimated to exist in the T_0 plant.

A DNA fragment homologous (59% identity at the amino acid level) to the WRKY4 transcription factor (DDBJ/EMBL/GenBank accession no. AF425835) from *A. thaliana* was found in the LB1g region, at 695–952 bp 5' upstream of the junction. The DNA sequence of LB1g, which included a *WRKY*-like gene, was deposited in the DDBJ/EMBL/GenBank under accession No. AB574419.

No other gene with significant homology was found by the BLAST search tool in the genomic DNA regions adjacent to the inserted T-DNA.

Figure 5. Schematic diagram of amplified fragments obtained in the analyses of T-DNA insertion loci in T_0. (**A**) T-DNAs connected with *P.somniferum* genome (10 types, eight sites), (**B**) T-DNAs connected in tandem (six types), (**C**) T-DNAs connected with orf13 fragments (four types).

(A)

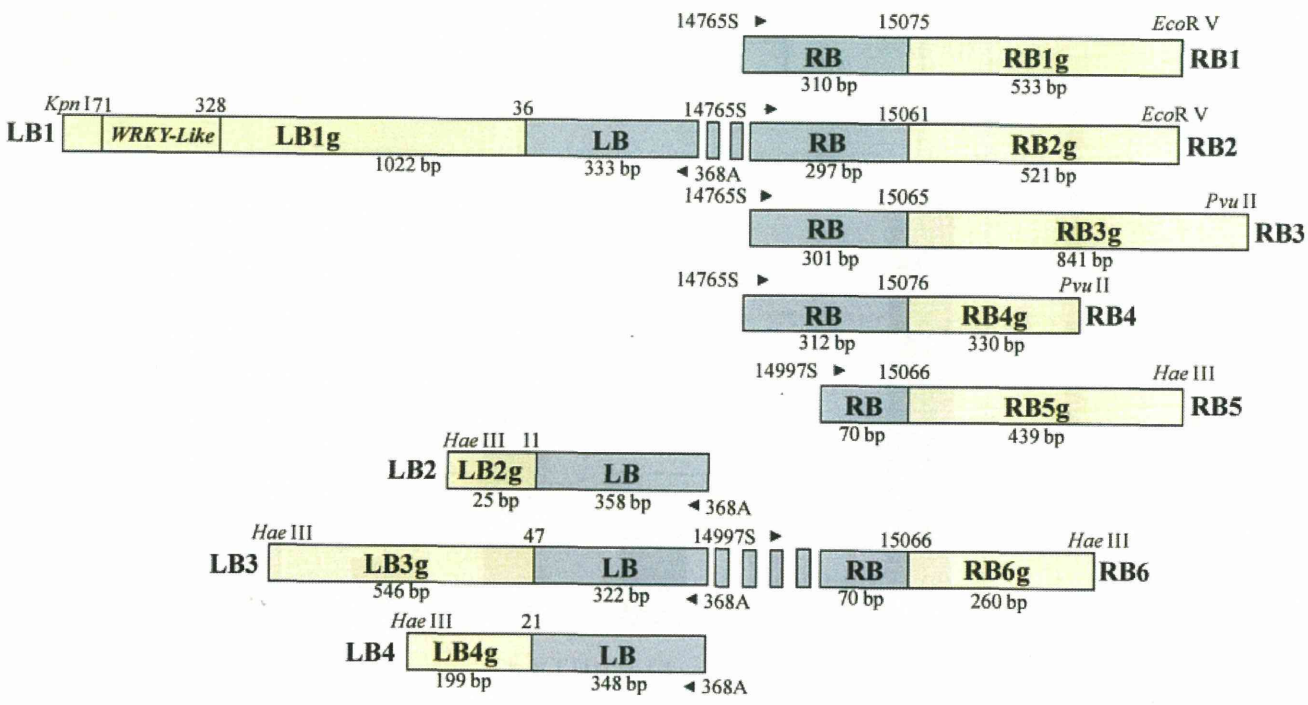
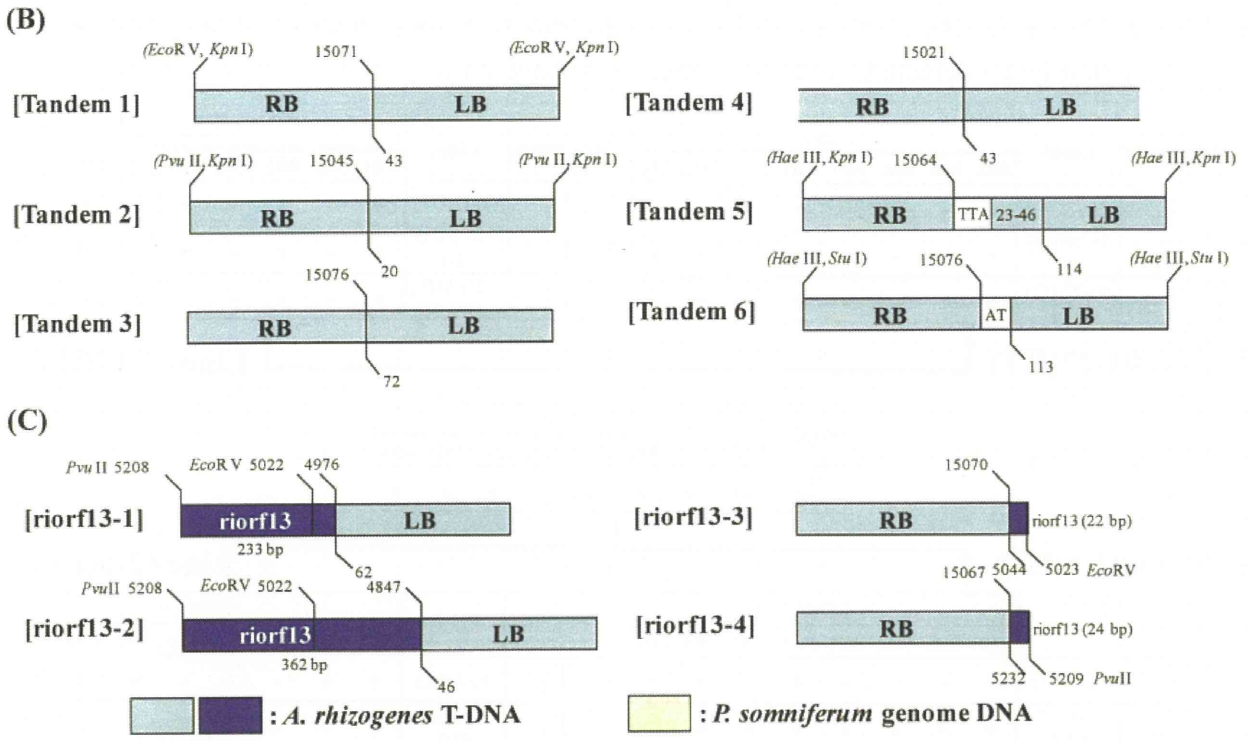


Figure 5. *Cont.*



Numbers correspond to the nucleotide positions in the T-DNA. Names of restriction enzymes used for the library construction and primers used for PCR are also shown. Fragment names with "g" indicate the fragments derived from the *P. somniferum* genome DNA. LB and RB correspond to the left and right border of T-DNA, respectively. Fragment "TTA" and "23–46" found in: "Tandem 5" were three nucleotide TTA and the complementary 24 bp sequence of the T-DNA left border (nucleotide number 23 to 46). Fragment "AT" found in "Tandem 6" was two bp nucleotides. "Riorf13" is the partial T-DNA fragment annotated as riorf13.

Type (B) includes six types of DNA fragments. RB and LB were connected in a tail-to head manner at different junctions, or short DNA fragments were sandwiched. The fragment "Tandem 3" was found only in the genome direct amplification of T-DNA borders.

Type (C) consists of four DNA fragments. Two of the fragments were made up of short partial fragments of T-DNA riorf13 attached to an LB, and the other two were made up of RB attached to a fragment of T-DNA riorf13. In summary, T-DNA border fragments found were at eight independent sites of T-DNA integration, six borders of T-DNAs connected in tandem, and four borders connected with T-DNA internal fragments.

As for the copy numbers, types (A) and (B) corresponded to eight and six copies of T-DNAs, respectively. In the case of type (C), LB and RB connected with riorf13 were possibly borders of independent T-DNAs or borders of the same T-DNA. Therefore, the copy number can be estimated as two at minimum to four at maximum. Finally, the T-DNA copy number in the PsM1-2 T_0 primary mutant could be estimated as 16 to 18.

2.4. Analysis of the Heredity Manner of T-DNA Inserted Loci

The PCR analysis over T-DNA border and the adjacent genomic DNA found in the IPCR and AL-PCR analyses revealed that several T-DNA inserted loci were eliminated by selfing (Figure 6).

Figure 6. Inheritance of the eight independent T-DNA insertion loci (RB1, RB2, RB3, RB4, RB5, LB2, LB3, and LB4) in the representative four lines of selfed progenies. (+: Insertion locus detected; -: insertion locus not detected.)

Progenies	Lines	T-DNA							
		RB1	RB2	RB3	RB4	RB5	LB2	LB3	LB4
T_1	#1-27(HT)	+	+	-	-	+	+	+	+
T_2	#1-27(HT) L#2	+	+	-	-	+	+	+	+
T_3	#1-27(HT) L#2 #1	+	+	-	-	+	+	+	+

Progenies	Lines	T-DNA							
		RB1	RB2	RB3	RB4	RB5	LB2	LB3	LB4
T_1	#2-17(HT)	+	-	+	-	+	+	+	+
T_2	#2-17(HT) #2-1	+	-	+	-	+	+	+	-
T_3	#2-17(HT) #2-1 #1	+	-	+	-	+	+	+	-

Line #1-27(HT) **Line #2-17(HT)**

Progeny	T-DNA							
	RB1	RB2	RB3	RB4	RB5	LB2	LB3	LB4
T_0	+	+	+	+	+	+	+	+

Line #2-1(LT) **Line #2-6(LT)**

Progenies	Lines	T-DNA							
		RB1	RB2	RB3	RB4	RB5	LB2	LB3	LB4
T_1	#2-1(LT)	+	-	+	-	+	+	+	-
T_2	#2-1(LT) #2-4	+	-	+	-	+	+	+	-

Progenies	Lines	T-DNA							
		RB1	RB2	RB3	RB4	RB5	LB2	LB3	LB4
T_1	#2-6(LT)	+	+	-	-	-	+	-	+
T_2	#2-6(LT) #2-2	+	+	-	-	-	+	-	+

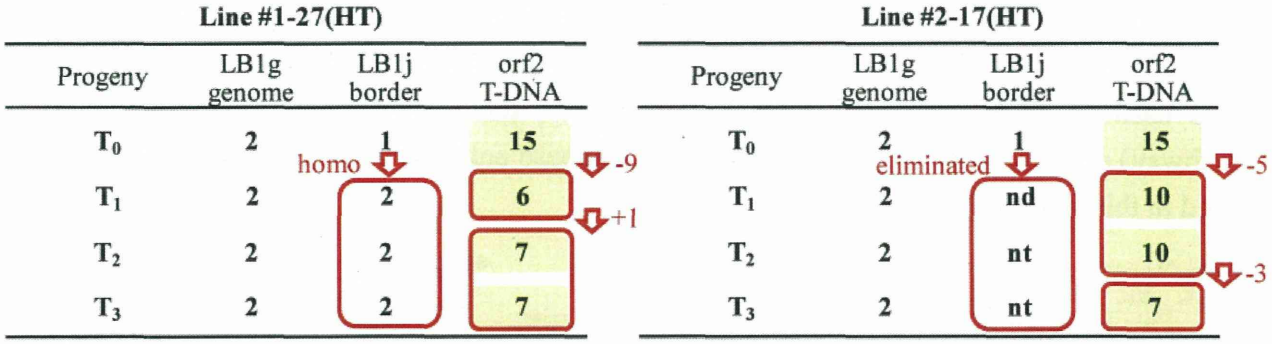
In the high thebaine line #1-27(HT), of the eight loci that were suggested to be independent T-DNA integration sites, RB3 and RB4 were eliminated in T_1 progeny; in addition, in the high thebaine line #2-17(HT), RB2, RB4 were eliminated in T_1 progeny and the additional elimination of LB4 was observed in T_2 progeny. On the other hand, with respect to the LT lines that showed low thebaine content at T_1 progeny, sites RB2, RB4, and LB4 were eliminated in #2-1(LT), and sites RB3, RB4, RB5, and LB3 were eliminated in #2-6(LT).

Notably thebaine content in these LT lines increased again in the T_2 progeny to 29.4% in #2-1(LT)#2-4 and to 21.1% in #2-6(LT)#2-2 (Table 2) without a change in the T-DNA insertion pattern (Figure 6). These results imply that none of the eight T-DNA integrated loci were indispensable for the high thebaine phenotype.

2.5. T-DNA Copy Number Analysis by Real-Time PCR

Standard curves for the quantification of the T-DNA copy number in T_0 and selected progenies were prepared for each target region, LB1g, LB1j and orf2. The formulae and correlation coefficients were as follows: LB1g: $y = -1.39\ln(x) + 23.82$ ($r^2 = 0.991$); LB1j: $y = -1.44\ln(x) + 23.38$ ($r^2 = 0.994$); and orf2: $y = -1.43\ln(x) + 23.75$ ($r^2 = 0.997$). The relative abundances of each region in the samples were calculated by these formulae from the value of Delta Rn. The relative abundances in whole numbers, when the abundance of LB1g was set as 2, were LB1g:LB1j:orf2 = 2:1:15 in T_0. And for T_1[#1-27(HT)] and its progenies, the abundances were as follows (in the order of LB1g:LB1j:orf2): T_1[#1-27(HT)], 2:2:6; T_2[#1-27(HT)L#2], 2:2:7; and T_3[#1-27(HT)L#2#1], 2:2:7. And for T_1[#2-17(HT)] and its progenies, the values were as follows: T_1[#2-17(HT)], 2:nd:10; T_2[#2-17(HT)#2-1], 2:nt:10; and T_3[#2-17(HT)#2-1#1], 2:nt:7 (nd: not detected; nt: not tested). These results are summarized in Figure 7.

Figure 7. Shift of the relative abundance of target regions LB1g, LB1j, and orf2 of T-DNA insertion locus LB1-RB2 analyzed for two selfed lines, #1-27(HT) and #2-17(HT) by quantitative real-time PCR.



The numbers in the column of orf2 represent the estimated copy number of T-DNA. (nd: Not detected; nt: not tested.) "Homo" indicates that the LB1-RB2 locus became homozygous at the T_1 progeny in #1-27(HT) and "eliminated" indicates that the LB1-RB2 locus was eliminated at the T_1 progeny in #2-17(HT).

For the abundance of LBj and orf2 in the #1-27(HT) series, the LB1-RB2 T-DNA insertion locus was estimated to become homozygous at the T_1 progeny, as indicated by the doubled abundance of LB1j in T_1. And the T-DNA copy number, estimated by the abundance of the orf2 region, was drastically decreased from 15 to six in T_1, then increased to seven in T_2 and kept at seven in T_3. These data imply that more than half of the total T-DNA copies were eliminated in the first selfing. For the #2-17 series, the LB1j region was not detected in T_1, which was consistent with the elimination of the LB1-RB2 T-DNA insertion loci in T_1 revealed by the T-DNA insertion loci analysis (Figure 6). The abundance of orf2 was decreased from 15 to 10 in T_1, and then decreased again from 10 to seven in T_3, which implies that more than half of the T-DNA copies in the #2-17(HT) series were also eliminated by repeated selfing.

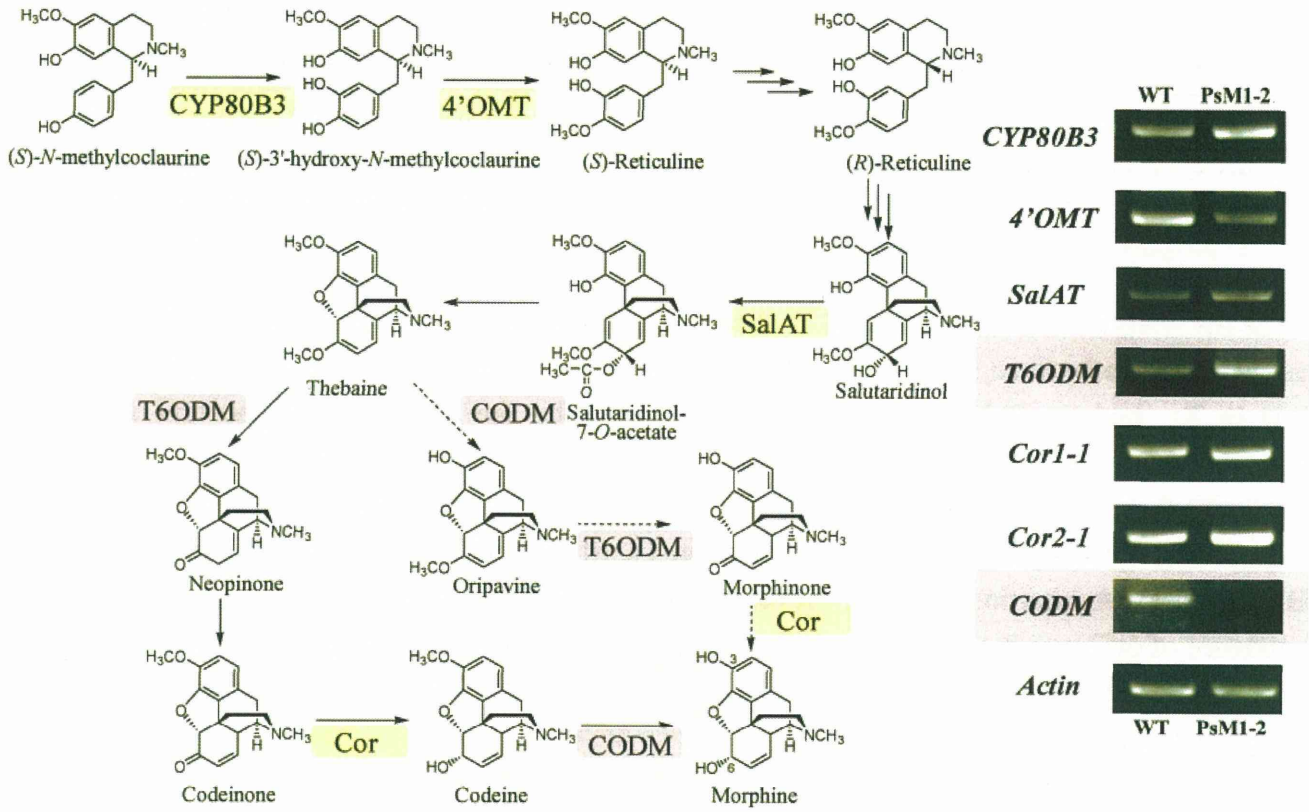
2.6. Expression Analyses on Morphine Biosynthetic Genes by RT-PCR

Firstly we tried to apply realtime-PCR for the expression analysis of morphine biosynthetic genes including *T6ODM*, *CODM* using the primer sequence reported by Hagel and Facchini [10]. However, prior to run the realtime-PCR, we found that PCR with these primers using our cDNA as a template gave multiple products. Although we have designed several primers, they could not make the PCR product as a single band. It may be attributed to the relatively high sequence homology of coding region among *T6ODM*, *CODM*, and *DIOX2*. Therefore we hired the semi-quantitative RT-PCR method for the expression analysis. To distinguish RT-PCR products between *T6ODM* and *CODM*, primers were designed to give different product size, *i.e.*, 549 bp for *T6ODM* and 411 bp for *CODM*.

Expression analysis on selected morphine biosynthetic genes downstream of (*S*)-*N*-methylcoclaurine revealed that the expression of *CODM* was completely diminished in PsM1-2 (Figure 8). On the other hand, the expression of *T6ODM* seemed to be slightly up-regulated in the PsM1-2 compared with the WT plant. Specific amplification of these two genes was confirmed by the comparison of the size of the bands and their calculated amplicon size. In PsM1-2, the expression levels of *CYP80B3* and *SalAT*

seem to be slightly higher than WT, whereas *4'OMT* seems to be down-regulated. No significant difference in the expression level of genes was observed between PsM1-2 and WT for *Cor1-1* or *Cor2-1*.

Figure 8. The morphine biosynthetic pathway downstream of (*S*)-*N*-methylcoclaurine with the results of expression analysis of selected morphine biosynthetic genes, *CYP80B3*, *4'OMT*, *SalAT*, *T6ODM*, *COR* (alleles *Cor1-1* and *Cor2-1*), and *CODM* by RT-PCR. Actin was used as an experimental control. Presumably, the pathway via oripavine (dotted pathway) does not exist in the *P. somniferum* Japanese cultivar "Ikkanshu" which we have used in this study [11,12].



2.7. Discussion

Morphological abnormalities, such as varied numbers of petals and splits on the boundary of petals, were frequently observed in the selfed progenies in the present study. However, no clear correlation was found between these morphological abnormalities and altered alkaloid compositions. Therefore, these findings were thought to be independent of the mutation in the secondary metabolism.

At the T_2 generation, difference between high thebaine line and low thebaine line which was obvious at T_1 generation, has disappeared. If the high thebaine phenotype is cause by the single mutation of the locus by T-DNA insertion, low thebaine phenotype should be dominant in the progeny plants. However, as observed in Figure 3, most of the progeny plants of low thebaine T_1 lines have gained high thebaine phenotype again, which indicates that the multiple loci are responsible for the high thebaine phenotype. For the reason of this phenomenon, it is also possible that, methylation or suppression has occurred in unstable manner on the alkaloid biosynthesis related genes caused by the multiple T-DNA insertion events.

The content of thebaine, which was the major alkaloid in the latex of the mature plants of the mutants, varied widely in the T_1 progeny. But by repeated selfing, in the T_3 progeny, although the maximum content of thebaine (10.9%) was not particularly high, the range of thebaine content was much narrower than that in the T_1 and T_2 progenies. When the value of CV (coefficient of variation: standard deviation/average value) for the thebaine content was compared among T_1, T_2, and T_3 progenies, it was 0.54 in T_1, 0.40 in T_2 (two HT lines combined), and 0.26 in T_3 (two lines combined). And the CV for the morphine content was 0.74 in T_1, 0.70 in T_2 (two HT lines combined), and 0.28 in T_3 (two lines combined). These lines of evidence indicate that the high thebaine (2.4-fold and 2.5-fold of WT in T_3 #1-27(HT)L#2 and #2-17(HT)#2-1, respectively) and low morphine (0.2-fold of WT in both T_3 #1-27(HT)L#2 and #2-17(HT)#2-1, respectively) phenotypes were stabilized by repeated selfing.

Analyses of the T-DNA integration sites and T-DNA copy number on the primary T_0 mutant revealed that at least eight integration sites exist and as many as 18 copies of T-DNAs were estimated to be integrated into the genomic DNA in a highly complicated manner. Considering the complexity of the T-DNA integration, the IPCR, AL-PCR, and real-time PCR methods employed in this study can be considered as the most suitable methods for T-DNA insertional analysis, and more suitable than Southern blotting, whose signals may be beyond interpretation in this context. The number of T-DNA copies in PsM1-2 was too large for the transgenes integrated by genetical transformation. The presence of high numbers of transgenic insertions can lead to poor expression of transgenes through silencing. In this study, we tried to simplify the T-DNA integration structure and stabilize the high thebaine phenotype, and then to gain insight into the genetic factors for the altered alkaloid composition by obtaining selfed progenies. The T-DNA integration sites in PsM1-2 were paired to be homozygous or dropped off by selfing, and finally became half of the T_0 in the selected T_3 progenies. Although it is possible that other T-DNA copies were not detected, no correlation was found between any of the T-DNA integration sites and the altered alkaloid composition, by considering these data together, a reduction in the T-DNA copy number seems to have resulted in the stabilization of the high thebaine phenotype. Although it is hard to confirm, there is also a possibility that genome reorganization independent of T-DNA insertion has occurred during shoot regeneration or long term maintenance of *in vitro* culture. As we have accomplished the stabilization of high thebaine phenotype by selfing up to T_3 generation, backcross experiment utilizing these selfed progeny plants is in progress.

In this study, the only gene homologous to the known gene found at the T-DNA integration loci was the *AtWRKY4* gene homologue found in the 5' upstream region of LB1g. As some type of WRKY transcription factor may function as a transcriptional regulator of benzylisoquinoline alkaloid biosynthesis in *Coptis japonica* Makino [13], the contribution of this locus to the altered alkaloid composition in the mutant was suspected. However, analysis of the T-DNA heredity manner indicated that the T-DNA insertion at LB1-RB2 region was not essential for the high thebaine phenotype.

The expression analyses on selected morphine biosynthetic genes, including two novel demethylases, *T6ODM* and *CODM*, between the *in vitro* shoot culture of the PsM1-2 mutant and seedlings of the WT plant revealed that the expression of *CODM* was fully suppressed in the mutant. Although the correlation between the transcript level of biosynthetic genes in young organs, such as seedling or *in vitro* shoot culture, and the alkaloid composition in the latex of mature plant needs to be clarified, the observed differences between the wild type plant and the mutant can be correlated to the alkaloid

composition difference in them (morphine was detected in the WT, however, almost no morphine in the mutant [8]).

Kinetic studies on recombinant T6ODM and CODM from *P. somniferum* [10] have revealed that oripavine is the most preferred substrate of T6ODM, followed by thebaine, while codeine is not accepted as a substrate. On the other hand, CODM showed a higher preference for codeine than thebaine. Considering the substrate preference of these two demethylases, thebaine can be accumulated solely only under the condition that the expression of both *T6ODM* and *CODM* is suppressed, and the suppression of *CODM* may result in accumulation of codeine. In actuality, however, a large amount of thebaine with a smaller amount of codeine is accumulated in the latex from mature plants of PsM1-2 mutants. This pattern of compounds detected in the mutant is similar to that of the *T6ODM*-silenced transformant by virus-induced gene silencing [10]. In contrast, the *CODM*-silenced transformant accumulates mainly codeine, together with smaller amounts of thebaine and morphine [10]. Although the alkaloid productivities of those transformants cannot be simply compared with PsM1-2, as the alkaloid composition varies highly even among the cultivars [14], it is assumed that suppression of CODM did not simply lead to the thebaine accumulation in PsM1-2. And it is also possible that in a Japanese cultivar that does not have the pathway from thebaine to morphine via oripavine [11,12], the substrate preferences of T6ODM and CODM differ from those of oripavine-producing cultivars.

As the regulation of opium alkaloid production in *P. somniferum* is highly complicated and varies among cultivars—and even among the developmental stages [15,16] or individual parts of a single plant [17]—further detailed studies on the molecular regulation of alkaloid production, such as expression analyses of *T6ODM* and *CODM* in the latex-producing capsule of PsM1-2, are required.

3. Experimental Section

3.1. Plant Materials

The wild type (WT) plant of *P. somniferum* L. used was the Japanese cultivar "Ikkanshu", and the *A. rhizogenes* strain MAFF03-01724 T-DNA insertion mutant line was PsM1-2 [8]. The *in vitro* culture of PsM1-2 used in this experiment was previously subjected to a single round of cryopreservation and regenerated to plantlet on Murashige-Skoog (MS) solid media [18] by the method described previously [19,20] with slight modifications.

3.2. Maintenance and Cultivation of Plant Materials

The WT plant seeds were obtained from the field-grown plants at the Research Center for Medicinal Plant Resources, Division of Tsukuba.

The PsM1-2 T_0 *in vitro* shoot culture was maintained on MS solid media at 20 °C under a 14 h light/10 h dark condition and then transplanted in soil in a 9 cm diameter pot and acclimatized in a phytotron in 60% relative humidity under a cycle of 16 h light at 20 °C and 8 h dark at 17 °C.

Seeds of T_1 plant obtained from the soil-cultivated plant of the PsM1-2 T_0 primary mutant were sown on the soil in a 15 cm diameter pot and cultivated in a greenhouse under a 16 h light/8 h dark cycle at 20 °C and 60% relative humidity. Plants were fertilized with 500-fold diluted Hyponex® (Hyponex Japan, Osaka, Japan) once a week.

T_2 seeds from the two lines of T_1 plants that showed high thebaine content and had abundant mature seeds were selected for cultivation of T_2 progeny. The cultivation conditions were the same as for T_1 plants.

T_3 seeds from two lines of T_2 plants with high thebaine content were germinated on rock wool with fertilization with 2,000-fold diluted Hyponex® in a greenhouse under a 16 h light/8 h dark cycle at 20 °C and 60% relative humidity. After one month, seedlings were transplanted onto the soil in a 9 cm diameter pot, and grown in the growth chamber under a 12 h light/12 h dark condition (short day condition) at 20 °C and 60% relative humidity. *Ca.* 80 days after sowing, the lighting was changed to a long day condition of 16 h light/8 h dark at 20 °C and 60% humidity for flowering. After transplanting, plants were fertilized with 500-fold diluted Hyponex® once a week.

For each experiment, WT plants were grown together as an experimental control. All self-pollination events were performed manually.

3.3. Phenotypic Observation of the PsM1-2 Mutants

Phenotypic parameters such as days to flowering, number of petals, appearance of splitting on the boundary of the petal, and the height of the aerial part at the seed-filling stage, were observed on each plant.

3.4. HPLC Analysis of Alkaloid Content in the Latex

The opium alkaloid content in the latex was analyzed by HPLC. Latex was collected from the capsule of either the WT or mutant *P. somniferum ca.* two weeks after flowering, by incising the capsule surface. Collected latex was dried at 50 °C. Approximately 5 mg of dried latex was measured accurately and subjected to alkaloid extraction by adding 5 mL of methanol followed by 30 min of sonication and mixing thoroughly using a tube mixer. After centrifugation at 20,000× *g* for 1 min, supernatant was applied to an Ultrafree-MC spin column (Millipore, Bedford, MA, USA) and centrifuged at 20,000× *g* for 1 min, and then 5 μL of the flow through was injected into an HPLC column. The HPLC conditions were as follows. HPLC instruments: Waters Alliance PDA System (separation module: 2795; photodiode array detector: 2996) (Waters, Milford, MA, USA). Column: TSK-GEL ODS100V (pore size 5 μm, ϕ4.6×250 mm) (Tosoh, Tokyo, Japan). Solvent system: CH_3CN (A), 10 mM sodium 1-heptanesulphonate (pH 3.5) (B). Solvent gradient (A%): 0 min 28%, 15 min 34%, 25 to 39 min 40%, 40 min 28%. Detection: UV 200 to 400 nm (spectrometric identification of compounds), UV 284 nm (quantitative analysis). Column temperature: 30 °C. Flow rate: 0.7 mL/min. HPLC data were collected and analyzed by an Empower system (Waters).

Alkaloid components were identified by the comparison of retention time and the UV spectra with authentic standards. Morphine hydrochloride and codeine phosphate were purchased from Takeda Pharmaceutical Company Limited (Osaka, Japan). Oripavine was a gift from Einar Brochmann-Hanssen (University of California, San Francisco, CA, USA). Magnoflorine iodide and jateorrhizine were gifts from Akira Ikuta (Science University of Tokyo, Japan). Reticuline and columbamine were gifts from Fumihiko Sato (Kyoto University, Japan). Isothebaine was isolated from *Papaver pseudo-orientale* (Fedde) Medw. by our group. Thebaine was a gift from Ruri Kikura-Hanajiri (National Institute of Health Sciences, Japan). Papaverine hydrochloride, noscapine hydrochloride, coptisine chloride, sanguinarine chloride, and berberine chloride were purchased from Wako Pure Chemical Industries (Osaka, Japan). Alkaloid contents were calculated as a weight percent of the dried latex (opium).