3.5. Genomic DNA Preparation from P. somniferum

Genomic DNA was prepared from *ca.* 100 μg of fresh leaves of selfed plants grown in the growth chamber, or from *ca.* 100 μg of whole *in vitro* plantlet of the PsM1-2 T_0 mutant, which mainly consisted of leaves and stems, by using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

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

The inverse-PCR (IPCR) method [21,22] and adaptor ligation PCR (AL-PCR) method were used for the analysis of the flanking unknown genome DNA sequence, adjacent to the inserted T-DNA. In this study, the Vectorette PCR method [23-25], an improved method of AL-PCR, was employed to reduce non-specific amplicons.

The genomic DNA library for each PCR method was constructed by digestion of genomic DNA by the appropriate restriction enzymes and self-ligation to form a circular DNA library, or an adaptor linker attached genome DNA library.

3.7. Genomic Library Construction for IPCR

Genomic DNA was digested with the restriction enzymes *Bam*HI, *Eco*RV, *Hae*III, *Kpn*I, *Pvu*II, *Ssp*I, or *Stu*I. Completely digested DNA was ligated by using a Fastlink® DNA Ligation Kit (AR Brown, Tokyo, Japan) to form a circular genome DNA library.

3.8. Genomic Library Construction for AL-PCR

The sequences of the adaptor oligo DNA and adaptor specific primers used in this study are listed in Supplementary Table 2. Two complementary oligo DNAs, AP-LS and AP-SS, were annealed to form an adaptor unit. Genomic DNA was digested with the restriction enzymes *Eco*RV, *Hae*III, *Pvu*II, *Ssp*I, or *Stu*I, which produce blunted ends. The completely digested DNA was ligated with adaptor units by using a Fastlink® DNA Ligation Kit to form an adaptor ligated genome DNA library.

3.9. IPCR and AL-PCR

Amplification of the target region was performed by the nested PCR method using TaKaRa Ex Taq™ DNA polymerase (Takara Bio, Shiga, Japan) under the following conditions. The combinations of PCR methods, template genome DNA libraries and primer sets are listed in Supplementary Table 1. T-DNA-specific primers were designed based on the DNA sequence of the T-DNA region of the *A. rhizogenes* plasmid pRi1724 (DDBJ/EMBL/GenBank accession no. AP002086). The first PCR conditions were as follows: primary denaturation at 94 °C for 5 min; followed by 30 cycles of 94 °C for 1 min, 42 °C for 2 min, and 72 °C for 3 min; with a final extension at 72 °C for 10 min. After PCR, the solution was held at 4 °C. The first PCR reaction solution was applied to a SUPREC™-02 filter (Takara Bio) to eliminate the primers and then used as a template for the second PCR. Second PCR conditions were as follows: primary denaturation at 94 °C for 5 min; followed by 30 cycles of 94 °C for 1 min, 48 °C for 2 min, and 72 °C for 3 min; with a final extension at 72 °C for 10 min. After PCR,

the solution was held at 4 °C. The product of the second PCR was gel purified and cloned into the sequencing vector pT7-Blue® (Novagen, Madison, WI). Propagated plasmid DNA was subjected to DNA sequencing using a BigDye® Terminator v3.1 Cycle Sequencing Kit and ABI PRISM® 3100—Avant Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan). Homology search was performed on T-DNA flanking genome DNA sequences with the BLAST tool at NCBI.

3.10. Direct Amplification of T-DNA Borders Connected in Tandem

PCR was performed on uncut genome DNA of T_0 to amplify the border region of T-DNAs connected in tandem. The primers used are listed in Supplementary Table 1. The PCR conditions were the same as for the IPCR.

3.11. Analyses of T-DNA Insertion Loci and Heredity Manner by PCR

The PCR method was employed to confirm the T-DNA integration loci on *P. somniferum* genome DNA and to analyze the heredity manner in the selfed progenies.

To find out the tally of the paired genomic regions found adjacent to the T-DNA left borders (LBs) and right borders (RBs) revealed by IPCR and AL-PCR analyses, PCR amplification was performed with the pair of genomic region-specific LB and RB (e.g., LB1g *vs.* RB2g) primers listed in Supplementary Table 3, under the following PCR conditions: primary denaturation at 94 °C for 5 min; followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; with a final extension at 72 °C for 10 min. After PCR, the solution was held at 4 °C. TaKaRa Ex Taq™ was used as the PCR polymerase. The PCR product was separated on agarose gel. The paired genomic regions, which gave a PCR product was judged as the single T-DNA integrated locus.

To judge whether or not the T-DNA integration loci were present in the selfed progenies, PCR amplification was performed between the genome region-specific primers listed in Supplementary Table 3 and T-DNA LB- or RB-specific primers (MAFF-226A or MAFF-14963S). PCR was performed under the following PCR conditions: primary denaturation at 94 °C for 5 min; followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; with a final extension at 72 °C for 10 min. After PCR, the solution was held at 4 °C. GoTaq® Green Master Mix (Promega, Madison, WI, USA) was used as the PCR polymerase. The PCR product was separated on agarose gel.

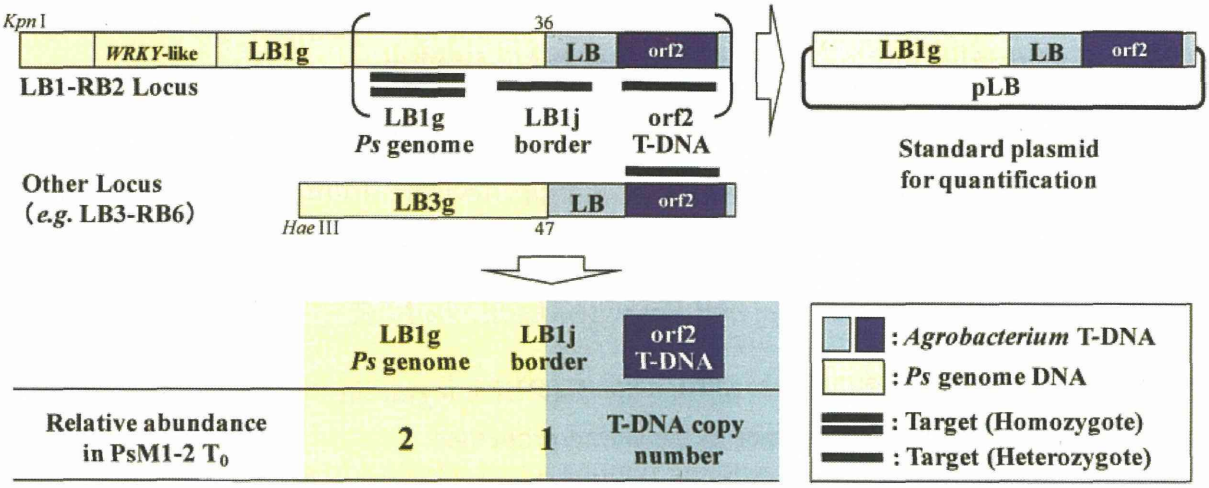
3.12. T-DNA Copy Number Analysis by Real-time PCR

The T-DNA copy number was analyzed by the quantitative real-time PCR method [26,27]. The strategy used for estimating the T-DNA copy number is as follows. When information of one of the integrated T-DNA sites was provided by T-DNA integrated loci analysis, and also, when the T-DNA integrated site was a single copy, the copy number of the integrated T-DNA could be calculated as a multiple of the relative abundance of standard DNA fragments, as shown in Figure 9.

This estimation method can be enacted under the hypotheses that (1) the genome DNA of PsM1-2 is diploid (2n = 22) [28], (2) all of the T-DNA is integrated into the host genome DNA in a heterozygous manner, and (3) one of the integrated T-DNAs for which both the LB and RB borders are known (e.g., the LB1-RB2 locus) is a single copy.

Under these hypotheses, by comparing the relative abundance of the T-DNA internal region (in this case, orf2), the T-DNA—*P. somniferum* genome junction region (LB1j), and the *P. somniferum* genome region (LB1g), we can calculate the inserted T-DNA copy number by fixing the abundance of LB1g as two.

Figure 9. Schematic diagram of the strategy of T-DNA copy number analysis by real-time PCR.



The LB1-RB2 locus whose LB and RB ends are confirmed was used as a standard. By setting the abundance of the genome DNA region (LB1g) of LB1 at a standard value of 2, the relative abundance of LB1j could be calculated as 1. The relative abundance of the orf2 region in the sample corresponded to the integrated T-DNA copy number. For the quantification, a standard plasmid pLB was constructed. "*Ps* genome" indicates the genome DNA of *P. somniferum*.

In our experiment, one of the T-DNA-integrated sites, LB1-RB2, which will be described in the Results section, was set as a standard. And for the quantification standard plasmid DNA, we constructed pLB1, which included, the LB1g, LB1j, and orf2 regions of the T-DNA. A DNA fragment with these three regions was amplified by PCR with the primers LB1-orf2-S (5'-CTC ATA AGC AGT GGT ATT GCT C-3') and LB1-orf2-A (5'-CGC ATT CAT GCG GTT ATG GAG-3') and KOD-Plus-DNA polymerase (Toyobo, Osaka, Japan) under the following PCR conditions: primary denaturation of 94 °C for 2 min; followed by 35 cycles of 94 °C for 15 s, 62 °C for 30 s, 68 °C for 90 s. After PCR, the solution was held at 4 °C. The amplified product was cloned into the pT7-Blue® vector (Novagen) and then propagated in *E. coli*. The quantitative standard plasmid DNA pLB1 and genome DNA prepared from the primary T_0 mutant and selected T_1, T_2, and T_3 progenies of the PsM1-2 mutant were diluted serially with the dilution buffer supplied with the real-time PCR reagent SYBR® Premix Ex Taq™ II (Perfect Real Time; Takara Bio). Real-time PCR was run using the target region-specific primers listed in Supplementary Table 4 with the real-time PCR reagent on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems Japan). The obtained data were analyzed using the supplied software (Applied Biosystems Japan) and the relative abundance of each target region was deduced from each Delta Rn value using standard curves. Standard curves for each target region were plotted with the plasmid concentration (fg/μL) on the x-axis and the Delta Rn on the y-axis. The curves showed good correlations. The relative abundances of each target region were calculated so that the abundance of the LB1g region was 2, and then rounded off to a whole number.

3.13. Actin Gene Amplification from P. somniferum

A fragment of actin cDNA was amplified by degenerate PCR using the forward primer 5'-AAR GCN AAY MGN GAR AAR ATG AC, and the reverse primer 5'-CCR TAN ARR TCY TTN CKD ATR TC, which were designed from the completely conserved regions of the amino acid sequences of other actins, such as *Arabidopsis thaliana* (*actin-1*: DDBJ/EMBL/GenBank accession No. M20016), *Nicotiana tabacum* (*actin*: X63603), and *Zea mays* (*Maz56*: U60514). cDNA synthesized from the total RNA of young seedlings of *P. somniferum* was used as a template for PCR. The manual hot-start procedure was used for the amplification. TaKaRa Ex Taq™ DNA polymerase was added after primary denaturation at 94 °C for 5 min, and then the following protocol was carried out in a GeneAmp2400 thermal cycler (Applied Biosystems Japan): 30 cycles of 94 °C for 1 min, 48 °C for 2 min, and 72 °C for 3 min; with a final extension at 72 °C for 10 min. After PCR, the solution was held at 4 °C. The amplified fragment was cloned into the pT7-Blue® vector followed by DNA sequencing. Two representative actin cDNA sequences, whose deduced amino acid sequences showed 92% and 95% identity to the *Arabidopsis actin-1*, were named *PsACT1* (AB574417) and *PsACT2* (AB574418), respectively.

3.14. Expression Analysis of the Morphine Biosynthetic Genes

The expression levels of selected morphine biosynthetic genes downstream of (*S*)-*N*-methylcoclaurine, *CYP80B3* (DDBJ/EMBL/GenBank accession no. AF134590 [29]), (*R,S*)-3'-hydroxy-*N*-methylcoclaurine 4'-*O*-methyltransferase (*4'OMT*; AY217333 [15]), *SalAT* (AF339913 [30]), *T6ODM* (GQ500139 [10]), *COR* (allele *Cor1-1*: AF108432; allele *Cor2-1*: AF108438 [31]), and *CODM* (GQ500141 [10]) in the WT plant and the PsM1-2 mutant were analyzed and compared by the reverse transcription PCR (RT-PCR) method.

Total RNA was prepared from the whole plants of two-week-old seedlings of field-grown WT *P. somniferum*, or from whole *in vitro* plantlet of the PsM1-2 T_0 mutant, which mainly consisted of leaves and stems, by using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. One microgram of total RNA samples was subjected to single-stranded cDNA synthesis by reverse-transcription with oligo-(dT) primer (RACE32: 5'-GAC TCG AGT CGA CAT CGA TTT TTT TTT TTT TT-3') [32] using Superscript® II Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Synthesized ss-cDNA was used as a template for PCR with the gene-specific primers listed in Supplementary Table 5. The PCR conditions were as follows: primary denaturation 94 °C for 5 min; followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; with a final extension at 72 °C for 10 min. After PCR, the solution was held at 4 °C. PCR products were separated on 1.0% agarose gel and signal intensities were observed. The actin gene *PsACT1* from *P. somniferum* was used as an experimental control.

3.15. Statistical Analysis

Values were expressed as the mean ± standard deviations (SD) and were analyzed by the Tukey-Kramer multiple comparison test using the statistical analysis system "R" software package [33]; a p value of less than 0.05 was considered significant.

4. Conclusions

By combining genetic and phenotypic analyses of the T-DNA insertional mutant PsM1-2 with selfing, we have succeeded in stabilizing the high thebaine phenotype in coordination with a reduction in the number of inserted T-DNA copies. Although the genetic mode of CODM suppression in *in vitro* plantlet and of the accumulation of thebaine still remain unknown, studies on this mutant and its progenies may provide new insights into the molecular basis of morphine biosynthesis, and could ultimately allow us to manipulate the biosynthesis of this compound at will.

Acknowledgments

We thank Naoko Tanaka and Naoko Onodera for their technical assistance. This study was supported in part by a grant from the Ministry of Health, Labor and Welfare of Japan.

Conflict of Interest

The authors declare no conflict of interest.

References

1. Frick, S.; Chitty, J.A.; Kramell, R.; Schmidt, J.; Allen, R.S.; Larkin, P.J.; Kutchan, T.M. Transformation of opium poppy (*Papaver somniferum* L.) with antisense berberine bridge enzyme gene (anti-bbe) via somatic embryogenesis results in an altered ratio of alkaloids in latex but not in roots. *Transgenic Res.* **2004**, *13*, 607–613.
2. Allen, R.S.; Millgate, A.G.; Chitty, J.A.; Thisleton, J.; Miller, J.A.C.; Fist, A.J.; Gerlach, W.L.; Larkin, P.J. RNAi-mediated replacement of morphine with the nonnarcotic alkaloid reticuline in opium poppy. *Nat. Biotechnol.* **2004**, *22*, 1559–1566.
3. Larkin, P.J.; Miller, J.A.; Allen, R.S.; Chitty, J.A.; Gerlach, W.L.; Frick, S.; Kutchan, T.M.; Fist, A.J. Increasing morphinan alkaloid production by over-expressing codeinone reductase in transgenic *Papaver somniferum*. *Plant Biotechnol. J.* **2007**, *5*, 26–37.
4. Frick, S.; Kramell, R.; Kutchan, T.M. Metabolic engineering with a morphine biosynthetic P450 in opium poppy surpasses breeding. *Metab. Eng.* **2007**, *9*, 169–176.
5. Allen, R.S.; Miller, J.A.; Chitty, J.A.; Fist, A.J.; Gerlach, W.L.; Larkin, P.J. Metabolic engineering of morphinan alkaloids by over-expression and RNAi suppression of salutaridinol 7-*O*-acetyltransferase in opium poppy. *Plant Biotechnol. J.* **2008**, *6*, 22–30.
6. Kempe, K.; Higashi, Y.; Frick, S.; Sabarna, K.; Kutchan, T.M. RNAi suppression of the morphine biosynthetic gene *salAT* and evidence of association of pathway enzymes. *Phytochemistry* **2009**, *70*, 579–589.
7. Millgate, A.G.; Pogson, B.J.; Wilson, I.W.; Kutchan, T.M.; Zenk, M.H.; Gerlach, W.L.; Fist, A.J.; Larkin, P.J. Analgesia: Morphine-pathway block in *top1* poppies. *Nature* **2004**, *431*, 413–414.
8. Yoshimatsu, K.; Shimomura, K. Transformation of opium poppy (*Papaver somniferum* L.) with *Agrobacterium rhizogenes* MAFF 03-01724. *Plant Cell Rep.* **1992**, *11*, 132–136.

9. Yoshimatsu, K.; Tono, K.; Shimomura, K. Cryopreservation of Medicinal Plant Resources—Retention of Biosynthetic Capabilities in Transformed Cultures. In Proceedings of the Japan International Research Center for Agricultural Sciences (JIRCAS)/International Plant Genetic Resources Institute (IPGRI) Joint International Workshop, Tsukuba, Japan, 20–23 October 1998; pp. S1–S6.
10. Hagel, J.M.; Facchini, P.J. Dioxygenases catalyze the *O*-demethylation steps of morphine biosynthesis in opium poppy. *Nat. Chem. Biol.* **2010**, *6*, 273–275.
11. Nielsen, B.; Röe, J.; Brochmann-Hanssen, E. Oripavine—A new opium alkaloid. *Planta Med.* **1983**, *48*, 205–206.
12. Brochmann-Hanssen, E. A second pathway for the terminal steps in the biosynthesis of morphine. *Planta Med.* **1984**, *50*, 343–345.
13. Kato, N.; Dubouzet, E.; Kokabu, Y.; Yoshida, S.; Taniguchi, Y.; Dubouzet, J.G.; Yazaki, K.; Sato, F. Identification of a WRKY protein as a transcriptional regulator of benzylisoquinoline alkaloid biosynthesis in *Coptis japonica*. *Plant Cell Physiol.* **2007**, *48*, 8–18.
14. Frick, S.; Kramell, R.; Schmidt, J.; Fist, A.J.; Kutchan, T.M. Comparative qualitative and quantitative determination of alkaloids in narcotic and condiment *Papaver somniferum* cultivars. *J. Nat. Prod.* **2005**, *68*, 666–673.
15. Facchini, P.J.; Park, S.-U. Developmental and inducible accumulation of gene transcripts involved in alkaloid biosynthesis in opium poppy. *Phytochemistry* **2003**, *64*, 177–186.
16. Yoshikawa, T.; Furuya, T. Morphinan alkaloid production by tissues differentiated from cultured cells of *Papaver somniferum* (1). *Planta Med.* **1985**, *51*, 110–113.
17. Weid, M.; Ziegler, J.; Kutchan, T.M. The roles of latex and the vascular bundle in morphine biosynthesis in the opium poppy, *Papaver somniferum*. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 13957–13962.
18. Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* **1962**, *15*, 473–497.
19. Yoshimatsu, K.; Yamaguchi, H.; Shimomura, K. Traits of *Panax ginseng* hairy roots after cold storage and cryopreservation. *Plant Cell Rep.* **1996**, *15*, 555–560.
20. Yoshimatsu, K.; Shimomura, K. Cryopreservation of *Panax* (Ginseng). In *Biotechnology in Agriculture and Forestry 50, Cryopreservation of Plant Germplasm II*; Towill, L.E., Bajaj, Y.P.S., Eds.; Springer-Verlag: Berlin/Heidelberg, Germany, 2002; pp. 164–179.
21. Ochman, H.; Gerber, A.S.; Hartl, D.L. Genetic applications of an inverse polymerase chain reaction. *Genetics* **1988**, *120*, 621–623.
22. Fladung, M. Gene stability in transgenic aspen (*Populus*). I. Flanking DNA sequences and T-DNA structure. *Mol. Gen. Genet.* **1999**, *260*, 574–581.
23. Arnold, C.; Hodgson, I.J. Vectorette PCR: A novel approach to genomic walking. *PCR Methods Appl.* **1991**, *1*, 39–42.
24. Spertini, D.; Beliveau, C.; Bellemare, G. Screening of transgenic plants by amplification of unknown genomic DNA flanking T-DNA. *Biotechniques* **1999**, *27*, 308–314.
25. Zheng, S.J.; Henken, B.; Sofiari, E.; Jacobsen, E.; Krens, F.A.; Kik, C. Molecular characterization of transgenic shallots (*Allium cepa* L.) by adaptor ligation PCR (AL-PCR) and sequencing of genomic DNA flanking T-DNA borders. *Transgenic Res.* **2001**, *10*, 237–245.

26. Bubner, B.; Baldwin, I.T. Use of real-time PCR for determining copy number and zygosity in transgenic plants. *Plant Cell Rep.* **2004**, *23*, 263–271.
27. Yang, L.; Ding, J.; Zhang, C.; Jia, J.; Weng, H.; Liu, W.; Zhang, D. Estimating the copy number of transgenes in transformed rice by real-time quantitative PCR. *Plant Cell Rep.* **2005**, *23*, 759–763.
28. Levy, A.; Milo, J. Genetics and breeding of *Papaver somniferum*. In *Poppy: The Genus Papaver*; Bernáth, J., Ed.; Harwood Academic Publishers: Chur, Switzerland, 1998; pp. 93–103.
29. Huang, F.-C.; Kutchan, T.M. Distribution of morphinan and benzo[*c*]phenanthridine alkaloid gene transcript accumulation in *Papaver somninferum*. *Phytochemistry* **2000**, *53*, 555–564.
30. Grothe, T.; Lenz, R.; Kutchan, T.M. Molecular characterization of the salutaridinol 7-*O*-acetyltransferase involved in morphine biosynthesis in opium poppy *Papaver somniferum*. *J. Biol. Chem.* **2001**, *276*, 30717–30723.
31. Unterlinner, B.; Lenz, R.; Kutchan, T.M. Molecular cloning and functional expression of codeinone reductase: The penultimate enzyme in morphine biosynthesis in the opium poppy *Papaver somniferum*. *Plant J.* **1999**, *18*, 465–475.
32. Frohman, M.A.; Dush, M.K.; Martin, G.R. Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 8998–9002.
33. The R Project for Statistical Computing. Available online: http://www.r-project.org/ (accessed on 15 January 2012).

危険性増す大麻

幻覚成分 40年前の11倍

厚生労働省の各地方厚生局麻薬取締部が全国で押収した乾燥大麻[1]について、幻覚作用のある化学成分の平均含有率を調べたところ、約40年前の野生大麻の約11倍に増えていることがわかった。関係機関は「以前よりもさらに危険性が増しており、呼吸困難やパニック、精神障害を引き起こす恐れがある」と注意を呼びかけている。

[1] 乾燥大麻　大麻草の葉や花の部分を採取して乾燥させた製品。煙草のように紙に巻いたり、パイプに入れたりして点火し、吸煙する。陶酔感や幻覚作用をもたらす成分が含まれ、大麻取締法で所持や譲渡が禁じられている。昨年、全国で同法違反で検挙された1648人のうち、約80％が初犯で、約54％が30歳未満だった。

大麻の雌花（厚生労働省提供）

雌花の自家栽培横行

厚労省が調べたのは、テトラヒドロカンナビノール（THC）という化学成分。全国の麻薬取締部が押収し、2010年度に刑事裁判を終えた事件の乾燥大麻6376袋（286・6キロ）のうち、押収から長時間たっておらず、含有率が正確に出やすい335袋を調査したところ、THCの平均含有率は11・2％だった。

一方、旧厚生省時代の1970年、当時、吸引に使われていた野生や衣服繊維用の大麻草を対象にした調査では、平均含有率は約1％だった。

厚労省によると、大麻には雄花と雌花があるが、雌花を受粉させないまま育てると、THCが増える特性がある。含有率が約11倍に増えた理由について、同省は、より幻覚作用の強い大麻を作ろうと、インターネットなどで密輸品の種子を購入し、雌花だけを自家栽培する方法が広がったためとみている。10年度に刑事裁判を終えた事件の乾燥大麻のうち、雌花は73％を占めた。

国連の世界薬物報告書によると、欧米よりTHC含有率が高い雌花が流通しているという。体内に吸引すると、中枢神経や心臓血管、呼吸器などほぼ全ての器官に影響を与えるうえ、青少年期に使用した場合、将来、統合失調症や精神病を発症するリスクが高まる。

「雌花売れる」実刑の男証言

警察庁によると、全国の警察が摘発した大麻の自家栽培も2000年の47件から、09年には312件まで増加した。

福岡県警が今年9月に大麻取締法違反容疑で逮捕した大分県佐伯市の男の自宅アパートからは、高さ約1・5㍍に成長した大麻草6鉢と、20㌢ほどの苗24本が見つかった。いずれも未受精の雌花とみられ、成長した大麻草から茎を切り取り、苗から育てていた。

熊本県益城町の山中や自宅で大麻草約800株を栽培したとして実刑判決を受けた男も、11月の福岡地裁での公判で、「雌花の乾燥大麻しか売れないので、改良を続けた」と証言した。

捜査関係者は「雌花の方が作用が強いのは、常習者の間で常識になっている」と警戒している。

24. 12. 31

読売 朝・夕