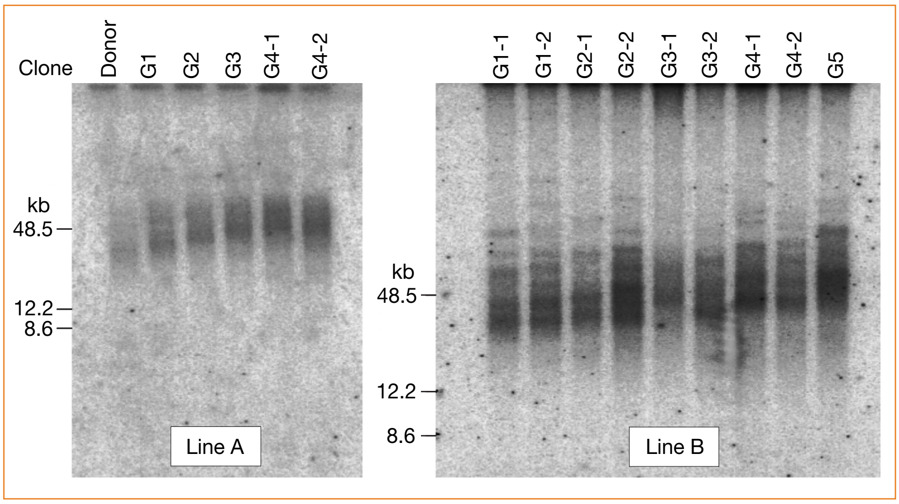


Figure 1 Telomere lengths in successive generations (G1–G5) of mice cloned from cumulus cells. Southern-blot analysis of terminal restriction-enzyme-cut fragments in five sequential generations shows that telomeres do not undergo incremental erosion in successive clonal generations. Genomic DNA isolated from peripheral-blood lymphocytes taken from representative animals from each generation was digested with the restriction enzyme *Hin*fI, resolved on a pulse field gel, transferred to a solid support and probed with a 5′-^{32}P-labelled $(T_2AG_3)_3$ oligonucleotide. Peripheral blood lymphocytes were sampled on the same day. Ages of mice (in months) were: in line A, donor, 18; G1, 16; G2, 14; G3, 12; G4, 9; G5, 9; in line B, G1, 15.5; G2, 13; G3, 11; G4, 9; G5, 7. Suffix numbers (G4-1 and G4-2, for example) identify different pups of each generation.

was repeated with cumulus cells from adult G1 mice as nucleus donors to produce the next clonal generation, G2, and so on. Table 1 summarizes the results obtained following the reconstruction of 3,920 enucleated oocytes.

Previously, about 2% of enucleated oocytes receiving a cumulus cell nucleus developed to live-born pups[1]. This value is comparable to the cloning efficiency for G1 in lines A (1.5%) and B (4.2%). However, the success rate dropped in successive cloned generations: line A did not produce a G5 clone from 670 reconstructed oocytes; in line B, the only live-born G6 clone was cannibalized by her foster mother, thereby terminating the line. Mouse lines A and B therefore represent totals of 9 and 26 clones from their respective donors. Placental size was consistently in the range previously reported for cloned mice[2] and did not increase in successive generations (data not shown).

Do sequentially cloned mice show signs of accelerated ageing? We assessed the behaviour of these mice and determined telomere lengths to assess organismal and cellular measures of ageing, respectively. We evaluated learning ability in the Morris water maze and Krushinsky tests, as well as strength and agility, and also used other assays designed to monitor signs of premature ageing, such as a decline in activity in the home cage and loss of coordination[4]. All cloned mice were, by these criteria, normal compared with age-matched controls (data not shown); the G5 mouse is alive and healthy at 1.5 years.

We measured telomere length in peripheral blood lymphocytes of clones G1–G6 by Southern-blot analysis of terminal restriction-enzyme-digested fragments (Fig. 1) and found no evidence of shortened telomeres in the cloned mice. In fact, our results show that the telomeres lengthen with each generation. As representative animals of each generation were sampled simultaneously, we cannot rule out an age-related contribution to this increase (with younger mice having longer telomeres). In addition, long telomeres in mice are optimally studied by means of different assays such as quantitative fluorescence *in situ* hybridization[5]. We have detected telomerase activity in cumulus cells (data not shown); it is therefore possible that telomeres in these cells are unusually long, resulting in offspring with concomitantly longer telomeres.

Shortened[6] and lengthened[7] telomeres have been reported in cloned livestock but, unlike ours, those experiments involved only a single round of cloning. Our results on sequentially cloned mice verify that telomere shortening is not a necessary outcome of the cloning process[8]. However, as only 1–2% of reconstructed oocytes yield live-born clones, the possibility of selection for donor nuclei with the longest telomeres cannot be excluded. Further investigation is required into the consequences of nuclear transfer on telomere length and lifespan.

Table 1 Effect of sequential cloning on full-term development

Line	*G1*	*G2*	*G3*	*G4*	*G5*	*G6*	*Total*
A	2/131 (1.5)	1/228 (0.4)	1/263 (0.4)	5/238 (2.1)	0/670 (0)	-	9/1,530 (0.6)
B	4/96 (4.2)	7/351 (2.0)	5/352 (1.4)	6/286 (2.1)	3/581 (0.5)	1/724 (0.1)	26/2,390 (1.1)

Successive generations are represented as G1, G2 and so on for two independent mouse lines, A and B. The number of pups born live after cumulus-cell nuclear transfer is compared to successfully reconstructed oocytes (pups/oocytes), with the corresponding percentages in parentheses. Significant χ^2 comparisons were derived for G4 and G5 from line A, G1 and G5, G6 from line B, and G2, G3, G4 versus G6 from line B ($P<0.05$).

Teruhiko Wakayama[*,†], Yoichi Shinkai[‡], Kellie L. K. Tamashiro[*], Hiroyuki Niida[§], D. Caroline Blanchard[||], Robert J. Blanchard[||], Atsuo Ogura[¶], Kentaro Tanemura[¶], Makoto Tachibana[‡], Anthony C. F. Perry[#], Diana F. Colgan[#], Peter Mombaerts[#], Ryuzo Yanagimachi[*]

[*] *Department of Anatomy and Reproductive Biology, John A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii 96822, USA*
[‡] *Department of Cell Biology, Institute for Virus Research, Kyoto University, Sakyo-Ku, Kyoto 606-8507, Japan*
[§] *Life Sciences Division, Lawrence Berkeley National Laboratory, University of California, Berkeley, Cailfornia 94720, USA*
[||] *Bekesy Laboratory of Neurobiology, University of Hawaii, Honolulu, Hawaii 96822, USA*
[¶] *Department of Veterinary Science, National Institute of Infectious Disease, Tokyo 162-8640, Japan*
[#] *The Rockefeller University, 1230 York Avenue, New York, New York 10021, USA*
[†] *Present address: The Rockefeller University, 1230 York Avenue, New York, New York 10021, USA*
e-mail: wakayat@mail.rockefeller.edu

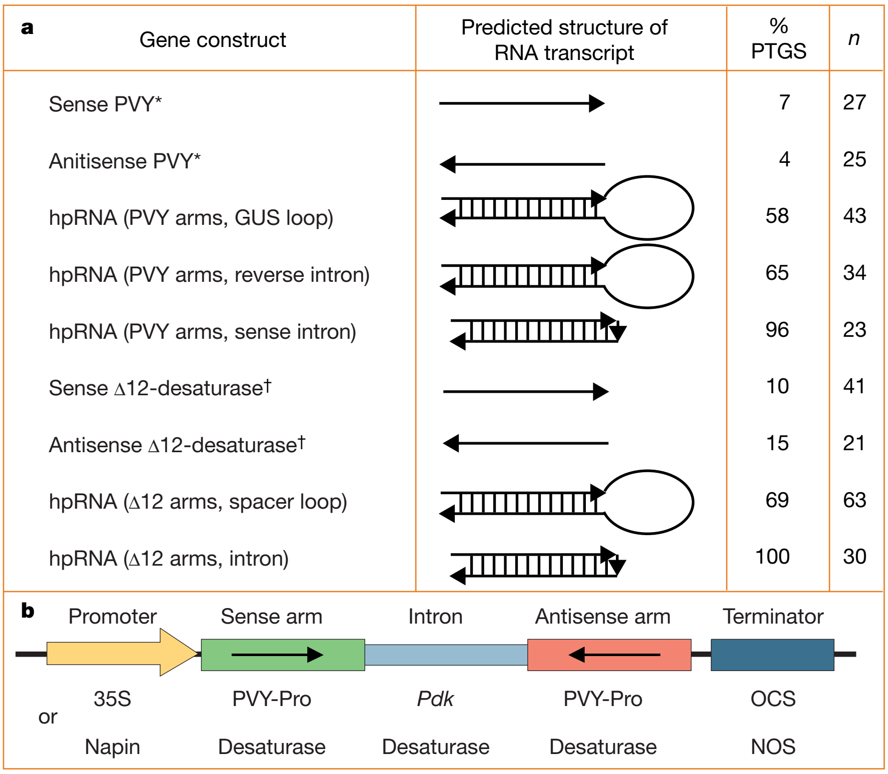
---

1. Wakayama, T., Perry, A. C. F., Zuccotti, M., Johnson, K. R. & Yanagimachi, R. *Nature* **394,** 369–374 (1998).
2. Wakayama, T. & Yanagimachi, R. *Nature Genet.* **22,** 127–128 (1999).
3. Wakayama, T., Rodriguez, I., Perry, A. C. F., Yanagimachi, R. & Mombaerts, P. *Proc. Natl Acad. Sci. USA* **96,** 14984–14989 (1999).
4. Tamashiro, K. L., Wakayama, T., Blanchard, R. J., Blanchard, D. C. & Yanagimachi, R. *Biol. Reprod.* **63,** 328–334 (2000).
5. Zijlmans, J. M. *et al. Proc. Natl Acad. Sci. USA* **94,** 7423–7428 (1997).
6. Shiels, P. G. *et al. Nature* **399,** 316–317 (1999).
7. Lanza, R. P. *et al. Science* **288,** 665–669 (2000).
8. Wilmut, I., Clark, J. & Harley, C. B. *Nature Biotech.* **18,** 599–600 (2000).

Gene expression

Total silencing by intron-spliced hairpin RNAs

Post-transcriptional gene silencing (PTGS), a sequence-specific RNA degradation mechanism inherent in many life-forms, can be induced in plants by transforming them with either antisense[1] or co-suppression[2] constructs, but typically this results in only a small proportion of silenced individuals. Here we show that gene constructs encoding intron-spliced RNA with a hairpin structure can induce PTGS with almost 100% efficiency when directed against viruses or endogenous



a Gene construct	Predicted structure of RNA transcript	% PTGS	*n*
Sense PVY*		7	27
Anitisense PVY*		4	25
hpRNA (PVY arms, GUS loop)		58	43
hpRNA (PVY arms, reverse intron)		65	34
hpRNA (PVY arms, sense intron)		96	23
Sense Δ12-desaturase†		10	41
Antisense Δ12-desaturase†		15	21
hpRNA (Δ12 arms, spacer loop)		69	63
hpRNA (Δ12 arms, intron)		100	30

Figure 1 Efficiency of induction of post-transcriptional gene silencing (PTGS) by different gene constructs and the predicted structure of RNA transcribed from the transgenes. **a,** PTGS efficiency measured for potato virus (PVY) and Δ12-desaturase genes as the percentage of independent transgenic plants immune to PVY and the percentage of plants with enzyme activity reduced by more than 20% compared with wild type, respectively. In the predicted structures of RNA transcripts, right- and left-pointing arrows represent sense and antisense orientation of sequences, respectively; small vertical arrows represent splice-junction sequences remaining after the intron has been spliced out. Vertical lines in the predicted structures indicate duplex formation. Asterisks, data from ref. 3; daggers, data from ref 7; hpRNA, hairpin RNA; *n*, number of independent transformants; GUS, β-glucuronidase. **b,** Design of intron-containing hairpin constructs. OCS, octopine synthase; NOS, nopaline synthase.

genes. These constructs could prove valuable in reverse genetics, genomics, engineering of metabolic pathways and protection against pathogens.

Induction of PTGS by co-suppression and antisense methods that target the Nia-protease (*Pro*) gene sequence of potato virus Y (PVY)[3] cause silencing in 7% and 4% of independent transformants, respectively; induction of PTGS in these tobacco plants (*Nicotiana tabacum*) manifests as immunity[3,4] to the virus.

Using principles we developed for silencing constructs that express double-stranded RNA and inverted-repeat RNAs[3], we made a construct encoding a single self-complementary hairpin RNA (hpRNA) of the *Pro* sequence. The construct contains sense and antisense *Pro* sequences flanking an 800-nucleotide spacer fragment derived from the *uidaA* (GUS) gene (Fig. 1a). About 60% (25/43) of the plants that are transformed with this construct, many of which contained a single transgene copy, were immune to the virus. The spacer fragment contributed to the stability of the perfect inverted-repeat sequences, but it was not required for the specificity of the PTGS (Fig. 1a).

To test the effect of removing the loop region of hpRNA, we replaced the spacer with an intron sequence (Fig. 1a, b). The intron sequence provides stability to the DNA, but is spliced out during pre-mRNA processing[5] to produce loopless hpRNA. As a control, we made a sister construct in which the intron sequence was inserted in the reverse, non-splicing, orientation. When transformed into tobacco, 22 of 34 (65%) reverse-intron plants were immune, a similar frequency to plants transformed with the GUS spacer construct. Amazingly, we found that 22 of 23 plants transformed with the construct containing the functional intron were immune to the virus.

To test whether this enhancement by the sense-intron construct was a general phenomenon, we made two hpRNA constructs against the endogenous Δ12-desaturase (*Fad2*) gene of *Arabidopsis*, which catalyses the conversion of oleic to linoleic acid in the seed[6,7]; one construct contained an intron and the other a non-intron spacer region. We found that 69% (44/63) of the transgenic plants with the non-intron spacer region construct showed PTGS of the Δ12-desaturase gene, but that 100% (30/30) of plants transformed with the intron construct showed silencing of this gene.

How does the presence of this intron enhance silencing efficiency? The process of intron excision from the construct by the spliceosome might help to align the complementary arms of the hairpin in an environment favouring RNA hybridization, promoting the formation of a duplex. Alternatively, splicing may transiently increase the amount of hairpin RNA by facilitating, or retarding, the hairpin's passage from the nucleus, or by creating a smaller, less nuclease-sensitive loop.

Our PVY constructs contained intron-2 from the *Pdk* gene of *Flaveria*[8], whereas the Δ12-desaturase construct contained intron-1 from the *Arabidopsis Fad2* gene (Fig. 1b). PVY constructs were controlled by the constitutive CaMV35S (ref. 9) promoter and produced hpRNA containing the PVY coding-region sequence (700 nucleotides); the desaturase gene construct used the seed-specific napin promoter[10] to produce hpRNA representing 120 nucleotides of the 3′-untranslated region of the Δ12-desaturase gene.

We believe that constructs encoding intron-hpRNA should efficiently induce PTGS for a wide range of genes in a variety of circumstances and could become as useful to plant biology as RNAi[11,12] is to the study of nematodes and *Drosophila*. The transgene design might also have application in organisms in which RNAi has been obtained by injection of double-stranded RNA.

Neil A. Smith, Surinder P. Singh, Ming-Bo Wang, Peter A. Stoutjesdijk, Allan G. Green, Peter M. Waterhouse
CSIRO Plant Industry, Canberra, ACT 2601, Australia
e-mail: peter.waterhouse@pi.csiro.au

1. Hamilton, A. J., Lycett, G. W. & Grierson, D. *Nature* **346,** 284–287 (1990).
2. Jorgensen, R. A. *Science* **268,** 689–691 (1995).
3. Waterhouse, P. M., Graham, M. W. & Wang, M.-B. *Proc. Natl Acad. Sci. USA* **95,** 13959–13964 (1998).
4. Waterhouse, P. M., Smith, N. A. & Wang, M.-B. *Trends Plant Sci.* **4,** 452–457 (1999).
5. Wang, M.-B., Upadhyaya, M. N., Brettel, R. I. S. & Waterhouse, P. M. *J. Genet. Breed.* **51,** 325–334 (1997).
6. Okuley, J. *et al. Plant Cell* **6,** 147–158 (1994).
7. Cartea, M. E., Migdal, M., Galle, A. M., Pelletier, G. & Guerche, P. *Plant Sci.* **136,** 181–194 (1998).
8. Rosche, E. & Westhoff, P. *Plant Mol. Biol.* **29,** 663–678 (1995).
9. Gleave, A. P. *Plant Mol. Biol.* **20,** 1203–1207 (1992).
10. Stalberg, K., Ellerstrom, M., Josefsson, L. G. & Rask, L. *Plant Mol. Biol.* **23,** 671–683 (1993).
11. Fire, A. *et al. Nature* **391,** 806–811 (1998).
12. Sharp, P. A. *Genes Dev.* **13,** 139–141 (1999).

brief communications is intended to provide a forum for short, topical reports of general scientific interest and for technical discussion of recently published material of particular interest to non-specialist readers. Priority will be given to contributions that have fewer than 500 words, 10 references and only one figure. Detailed guidelines are available on *Nature*'s website (www.nature.com) or on request from **nature@nature.com**