lation with anti-CD28 enhanced NFATc nuclear accumulation (Fig. 4B), in keeping with the finding that T_H2 cytokine induction in wild-type T_H cells requires costimulation (Fig. 2C). In contrast, anti-CD3 treatment alone led to an increase in nuclear NFATc in *Jnk1*$^{-/-}$ T_H cells and a decrease in cytoplasmic NFATc (Fig. 4, A and B), consistent with the high T_H2 cytokine production by CD3-activated *Jnk1*$^{-/-}$ cells (Fig. 2C). The enhanced accumulation of nuclear NFATc in *Jnk1*$^{-/-}$ T_H cells was observed in cells 8, 24, and 48 hours after stimulation, but was not observed in nonactivated cells (*10*). NFATc accumulation was specific because the amount of nuclear NFATp, a proposed negative regulator of T_H2 cytokine genes (*21*), was the same in wild-type and *Jnk1*$^{-/-}$ cells (Fig. 4A). Enhanced nuclear accumulation of NFATc in *Jnk1*$^{-/-}$ T cells was not blocked by anti–IL-4 (Fig. 4A); hence, increased IL-4 production and NFATc nuclear localization is intrinsic to T cell receptor signaling and is not secondary to IL-4 production. Because NFATc can bind to the IL-4 promoter and is required for IL-4 production and T_H2 differentiation (*20, 22*), the greatly enhanced amount of nuclear NFATc could account for the increased IL-4 production in CD3-activated *Jnk1*-deficient mice.

The mechanism by which JNK1 negatively regulates NFATc nuclear accumulation remains to be resolved. The isoform NFAT4 is phosphorylated and negatively regulated by JNK, leading to nuclear exclusion (*23*). This regulation appears to be specific to the NFAT4 isoform; evidence for JNK regulation of NFATc was not reported (*23*). An indirect mechanism may therefore account for the altered regulation of NFATc in *Jnk1*$^{-/-}$ T_H cells. NFATc and NFATp can bind to the IL-4 promoter NFAT sites (*22*). Both *Jnk1* and *NFATp* knockout mice have enhanced T cell proliferation and T_H2 cytokine production (*21, 24*), precisely the opposite of the NFATc knockout. It is therefore possible that these two NFAT factors antagonize each other in the regulation of the IL-4 gene. The apparent similarity between *NFATp*$^{-/-}$ and *Jnk1*$^{-/-}$ phenotypes supports a functional linkage between JNK1 and NFAT.

Our results further reveal a novel mechanism by which TCR signaling negatively regulates T_H2 cytokines through JNK1. Positive and negative regulation of JNK1 activity may affect the decision of T_H cells to differentiate into T_H1 or T_H2 effectors, and therefore may affect the type of immune response that is initiated. The function of JNK1 demonstrated in this study is distinct from that of JNK2, which is required for IFN-γ production in T_H1 cells (*14*). Moreover, the related p38 mitogen-activated protein kinase pathway is T_H1 specific and drives IFN-γ transcription (*25*). Together, these pathways potentiate the T_H1 response and provide a potential target for pharmaceutical intervention.

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10. C. Dong, D. D. Yang, M. Wysk, A. J. Whitmarsh, R. J. Davis, R. A. Flavell, unpublished data.
11. The data are shown at *Science* Online (www.sciencemag.org).
12. Total spleen cells or purified CD4 T cells were stimulated as triplicates with Con A (2.5 μg/ml), plate-bound anti-CD3 with or without anti-CD28 (plates were precoated with antibody at 10 μg/ml). IL-2 production was measured by enzyme-linked immunosorbent assay (ELISA; Pharmingen) 24 hours after stimulation. Proliferation was assayed after 3 days of treatment by adding [^{3}H]thymidine to the culture for the last 8 hours. At day 4, the supernatant of stimulated cells was removed and T_H cytokine production was measured by ELISA. In activation-induced cell death experiments, CD4 T cells were stimulated with Con A for 4 days, extensively washed, and restimulated with immobilized anti-CD3 for 48 hours. Apoptosis was determined by staining the cells with 7-aminoactinomycin D (7-AAD) and Annexin V (Pharmingen); dead cells were scored as Annexin$^+$ 7-AAD$^+$. The data are shown at *Science* Online (www.sciencemag.org).
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Eight Calves Cloned from Somatic Cells of a Single Adult

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Eight calves were derived from differentiated cells of a single adult cow, five from cumulus cells and three from oviductal cells out of 10 embryos transferred to surrogate cows (80 percent success). All calves were visibly normal, but four died at or soon after birth from environmental causes, and postmortem analysis revealed no abnormality. These results show that bovine cumulus and oviductal epithelial cells of the adult have the genetic content to direct the development of newborn calves.

Nuclear transfer is an efficient technique for assessing the developmental potential of a nucleus and for analyzing the interactions between the donor nucleus and the recipient cytoplasm. In amphibians, successful nuclear transfer was first reported by Briggs and King who used blastula cells for nuclear transfer to oocytes, which proceeded to develop into

tadpoles (*1*) and later juvenile frogs (*2*). Other cell types, including germ cells and somatic cells from tadpoles, have also been shown to have developmental totipotency (*3*): their nuclei directed the formation of fertile amphibians. However, despite extensive studies in amphibians, progeny could not be generated from adult cell nuclei (*3*). This obstacle was recently overcome in sheep (*4*) and mice (*5*), and nuclei from fetal fibroblast cells have directed the formation of lambs (*4, 6*) and calves (*7*). Wakayama *et al.* (*5*) used nuclear transfer to produce fertile mice from cumulus cells collected from metaphase II oocytes. Here, we report cloning of calves at a high rate using cumulus cells and oviductal epithelial cells that were passaged several times in vitro.

Oviducts and ovaries used as the donor nuclear source were obtained from a local slaughterhouse from a single cow of Japanese beef cattle in an unknown stage of the estrous cycle. Cumulus cells from ovarian oocytes at the germinal vesicle stage and oviductal epithelial cells (*8, 9*) were collected and cultured for several passages (*10*), and cells quiescent in the G_0-G_1 phase by serum starvation for 3 to 4 days (*4, 11*) were used for nuclear transfer (*12*). The characteristics of donor cells were determined by labeling with vimentin and cytokeratine (Fig. 1).

Forty-seven percent of the enucleated oocytes fused with cumulus cells and 63% did so with oviductal epithelial cells (Table 1). Among these constructs, 37 cumulus and 88 oviductal nuclear transplants were selected for culture in vitro for 8 to 9 days, by which time 49% of the cumulus-derived and 23% of the oviductal-derived nuclear transplants had developed into blastocysts. A total of 10 blastocysts originating from both cell types were nonsurgically transferred into surrogate cows at day 7 or 8 after the onset of estrous. Six blastocysts derived from cumulus cells were transferred into three females, and four from oviductal cells were placed into two females. All five females became pregnant. Two of the three surrogates containing cumulus nuclear transplants and one of the two with oviductal transplants had multiple pregnancies. Of the 10 blastocysts transferred to cows, 8 cloned female fetuses completed gestation and were born (Table 2). Calves OVI-1, -2, CUM-3, -4, -5, -6, -7, and OVI-8 were delivered 242, 242, 266, 267, 267, 276, 276, and 287 days of gestation, respectively (OVI and CUM indicate origin from oviductal or cumulus cells). All calves were born vaginally except calf OVI-8, which was delivered by cesarean section because of dystocia. The average length of pregnancy of Japanese beef cattle with a female fetus is 286.6 ± 0.9 days and the average body weight at birth is 27.0 ± 0.8 kg. The pregnancy period is often shorter when there are two fetuses. The calves of OVI-1 and OVI-2 were born prematurely.

Four of the eight calves died. Postmortem analysis did not reveal any abnormality; however, environmental factors appeared to account for their deaths. Calf CUM-3 died 3 days after birth from pneumonia apostematosa stemming from heatstroke, CUM-4 and -5 died just after birth from drawing in superfluous amniotic fluid, and OVI-8 died at birth from dystocia and delayed delivery. The other four calves were healthy. In addition, most surrogate mothers showed no or few symptoms of parturition such as labor pains and mammary development. On 1 November 1998, OVI-1 and -2 calves were 120 days old and CUM-6 and -7 calves were 85 days old. The results of microsatellite-typing (*13*) indicated that the genomes of the cloned calves were identical to those of the donor cells, and different from those of the surrogate mothers (Table 3).

Nuclear transfer of adult somatic cells from farm animals is the most efficient technique for obtaining large numbers of genetically identical animals. Although preimplantation embryonic cells and fetal fibroblasts are also useful for cloning, the economic potential of the donor is not predictable. In contrast, adult somatic cells can be selected from animals already proven to be ideal milk or meat producers. In particular, cumulus cells are especially appropriate for cloning females, because they can be easily obtained without injury to the animals.

In our study, the percentage of nuclear transplants developing into blastocysts was quite high (23% from oviductal cells and 49% from cumulus cells) compared with that of bovine fetal fibroblasts (12%) reported by Cibelli *et al.* (*7*). Our higher efficiency may relate to our culture system in which 30% of the control oocytes matured and fertilized in vitro developed into blastocysts (*14*). Thus, our nuclear transplants were about equal to the controls in developmental ability to the

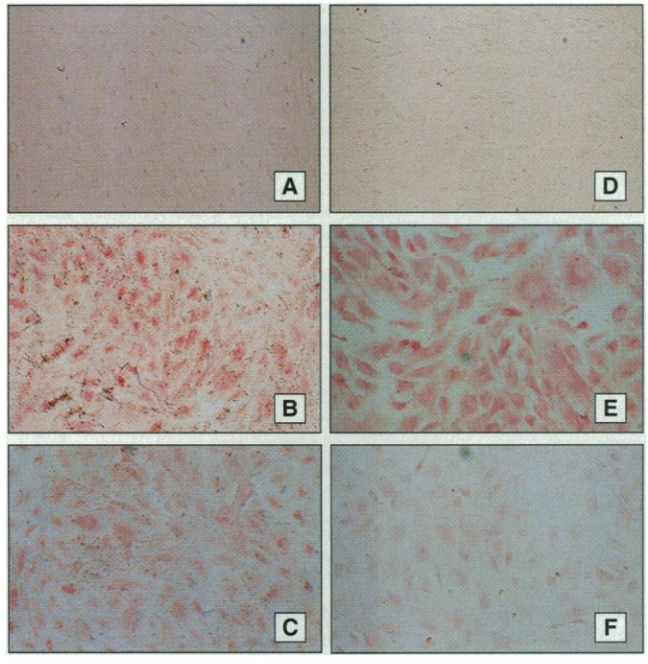

Fig. 1. Labeling of bovine oviductal (**A** to **C**) and cumulus (**D** to **F**) cells with vimentin B and E and cytokeratin (C and F). Panels (A) and (D) are negative controls. All oviductal epithelial cells were visually positive for a marker of epithelial cells, cytokeratin (C) (detected with rabbit antiserum to keratin), and for vimentin (B) (detected with rabbit antibody to vimentin) (*19*). All cumulus cells were also visually positive for vimentin (E) and cytokeratin (F), though the latter was very weak. Original magnification, × 100.

Table 1. Developmental potential of somatic nuclear transplants in vitro.

Origin of donor cells	No. of oocytes		No. of oocytes developed to			
	Fused/total	Cultured	Two-cell	Eight-cell	Morula	Blastocyst
Cumulus	47/99	37	31	25	21	18
Oviduct	94/150	88	77	58	39	20

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blastocyst stage. Furthermore, the quality of the nuclear transplant blastocysts was evidenced by the fact that they had normal cell numbers (69 to 114 cells) (*14*).

The high percentage of nuclear transplant embryos developing to term may be due to a number of factors. First, both donor cell populations maintained an apparent normal karyotype during the in vitro culture before use for nuclear transfer (*15*). Second, nucleo cytoplasmic interactions might be more compatible in this bovine experiment than in previous mouse experiments where the genetic type of the donor nucleus was critically important for later development (*16*). Third, although it was hypothesized that the donor cytoplasm of some somatic cell types might interfere with the development of nuclear transplants (*5*), the cumulus cytoplasm used in this study may have been compatible with the oocyte cytoplasm. The precursor cells of cumulus cells were connected by cytoplasmic bridges of microvilli and processes, through which cytoplasmic factors were exchanged. This exchange of factors might account for the higher percentage of nuclear transplant blastocysts from cumulus cell (49%) compared with oviductal cells (23%). Although, the telomerase activity of bovine cumulus cells is unclear, human cumulus cells, known to exhibit telomerase activity (*17*), might suffer fewer aging affects than other cell types and serve as an ideal adult donor cell for cloning. Fourth, twinning all embryos may have improved the survival rates of the embryos.

A problem for investigation concerns the cytoplasmic contribution of the oocyte to the properties of the clone. Bovine ovaries are often obtained from a slaughterhouse and the genetic background of the oocytes is unknown. In mice, cytoplasmic factors do affect the phenotype of nuclear transplants (*16*), but whether the effect stems from mitochondrial or maternal gene products is unknown. Two technical factors regarding the donor cells also require consideration, namely, freezing and the cell cycle stage. Large-scale cloning requires freezing of the donor cells. In our study, both donor cell types were freshly prepared and used before freezing. Although freezing of donor cells does not affect the in vitro development of nuclear transplants (*14*), the later developmental potential of such transplants is unknown. As cells are often damaged during freezing and thawing, this process should be carefully examined.

The application of somatic cell nuclear transfer to animal breeding poses many unanswered questions. Future studies are needed to reduce the death rate from environmental causes and also to reveal whether surviving calves grow normally into fertile adults. The low survival rate of calves might also be in part due to an epigenic component resulting from cloning and related procedures such as culture conditions, because the previous study on cloning bovine by nuclear transfer of embryonic nuclei reported similar postnatal problems (*18*). Whether these problems were caused by the nuclear transfer procedure or other factors is not known. Also, yet to be determined is whether other adult cell types can be reprogrammed to direct the development of fertile animals.

Table 2. Calves cloned from somatic cells. OVI and CUM designate the origin of the donor cells: oviduct and cumulus cells, respectively.

Calf number	Born at day	Weight at birth (kg)	Status
OVI-1	242	18.2	Living
OVI-2	242	17.3	Living
CUM-3	266	32.0	Dead (day 3)
CUM-4	267	17.3	Dead (day 0)
CUM-5	267	34.8	Dead (day 0)
CUM-6	276	23.0	Living
CUM-7	276	27.5	Living
OVI-8	287	30.1	Dead (day 0)

Table 3. DNA microsatellite analysis. The values indicate the fragment size in base pairs. DIK024, AG223, DIK069, DIK089, AG035, AG233, AG053, DIK106, DIK096, DIK020, DIK097, AG310, DIK102, AG119, DIK039, AG133, AG140, AG273, AG147, DIK010, AG160 and DIK068 are on the chromosome 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 17, 19, 20, 21, 22, 23, 24, 26, and 28, respectively. ND, not determined.

	DIK024	AG223	DIK069	DIK089	AG035	AG233	AG053	DIK106	DIK096	DIK020	DIK023	DIK097
OVI cells	234, 240	87, 87	160, 164	81, 90	166, 166	186, 186	285, 289	ND	251, 256	242, 242	100, 100	197, 201
CUM cells	234, 240	87, 87	160, 164	81, 90	166, 166	186, 186	285, 289	ND	251, 256	242, 242	100, 100	197, 201
OVI-1	234, 240	87, 87	160, 164	81, 90	166, 166	186, 186	285, 289	ND	251, 256	242, 242	100, 100	197, 201
OVI-2	234, 240	87, 87	160, 164	81, 90	166, 166	186, 186	285, 289	ND	251, 256	242, 242	100, 100	197, 201
mother	241, 243	82, 82	155, 164	88, 98	166, 166	192, 192	287, 292	ND	248, 248	242, 242	93, 108	192, 197
CUM-3	234, 240	87, 87	160, 164	81, 90	166, 166	186, 186	285, 289	ND	251, 256	242, 242	100, 100	ND
mother	233, 237	82, 82	166, 166	93, 98	166, 168	192, 192	287, 290	ND	250, 254	241, 241	93, 95	ND
CUM-4	234, 240	87, 87	160, 164	81, 90	166, 166	186, 186	285, 289	ND	251, 256	242, 242	100, 100	ND
CUM-5	234, 240	87, 87	160, 164	81, 90	166, 166	186, 186	285, 289	ND	251, 256	242, 242	100, 100	ND
mother	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
CUM-6	234, 240	87, 87	160, 164	81, 90	166, 166	186, 186	285, 289	ND	251, 256	ND	100, 100	ND
CUM-7	234, 240	87, 87	160, 164	81, 90	166, 166	186, 186	285, 289	ND	251, 256	242, 242	100, 100	ND
mother	233, 239	82, 82	154, 154	85, 93	166, 166	187, 192	289, 289	ND	244, 254	230, 232	84, 95	ND
OVI-8	234, 240	87, 87	160, 164	81, 90	166, 166	186, 186	285, 289	ND	251, 256	242, 242	100, 100	ND
mother	237, 248	82, 82	158, 160	87, 97	166, 168	185, 185	289, 289	ND	254, 254	243, 250	90, 99	ND

	AG310	DIK102	AG119	DIK039	AG133	AG140	AG273	AG147	DIK010	AG160	DIK068
OVI cells	ND	ND	233, 246	188, 194	135, 139	150, 156	114, 114	194, 211	185, 195	246, 246	147, 153
CUM cells	ND	ND	233, 246	188, 194	135, 139	150, 156	114, 114	194, 211	185, 195	246, 246	147, 153
OVI-1	ND	ND	233, 246	188, 194	135, 139	150, 156	114, 114	194, 211	185, 195	246, 246	147, 153
OVI-2	ND	ND	233, 246	188, 194	135, 139	150, 156	114, 114	194, 211	185, 195	246, 246	147, 153
mother	ND	ND	233, 233	188, 194	135, 144	146, 148	100, 114	186, 191	185, 192	239, 241	146, 146
CUM-3	ND	ND	233, 246	188, 194	135, 139	150, 156	114, 114	194, 211	185, 195	246, 246	147, 153
mother	ND	ND	233, 233	ND	135, 149	149, 156	100, 110	192, 200	192, 196	243, 246	143, 149
CUM-4	ND	ND	233, 246	188, 194	135, 139	150, 156	114, 114	194, 211	185, 195	246, 246	147, 153
CUM-5	ND	ND	233, 246	188, 194	135, 139	150, 156	114, 114	194, 211	185, 195	ND	147, 153
mother	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
CUM-6	ND	ND	233, 246	188, 194	135, 139	150, 156	114, 114	ND	185, 195	246, 246	147, 153
CUM-7	ND	ND	233, 246	188, 194	135, 139	150, 156	114, 114	ND	185, 195	246, 246	147, 153
mother	ND	ND	229, 233	184, 188	137, 149	149, 150	110, 114	191	194, 196	238, 244	146, 147
OVI-8	ND	ND	233, 246	188, 194	135, 139	150, 156	114, 114	ND	185, 195	246, 246	147, 153
mother	ND	ND	229, 233	194, 194	135, 149	147, 149	101, 114	185, 200	192, 195	240, 240	147, 147

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11. Cells cultured in 0.5% or less FBS for longer than 3 days attained a quiescent state (*14*).
12. For nuclear transfer, in vitro–matured oocytes were enucleated at 22 to 24 hours after maturation. A single donor cell was electrically fused with an oocyte immediately after enucleation with two pulses of 150 v/mm of dc for 25 μs in Zimmerman fusion medium. Pulses were repeated twice with an interval

of 15 min until fusion occurred. Fused oocytes were again electrically stimulated (20-v/mm dc pulses for 20 μs) to ensure activation. Nuclear transplant oocytes were immediately treated with cyclohexamide (10 μg/ml) in CR1-aa medium [C. F. Rosenkraus and N. L. First, *Theriogenology* **35**, 266 (abstr.) (1991)] with 3 mg of bovine serum albumin (fatty acid free) for 5 to 6 hours. After treatment, the oocytes were cultured in cyclohexamide-free medium. On day 3 (day 1 being the day of nuclear transfer), the nuclear transplant embryos were transferred to dishes containing CR-1aa medium supplemented with 10% FBS and mouse fetal fibroblast cells pretreated with mitomycin C (10 μg/ml) for 2.5 hours. On days 8 and 9 of in vitro culture, visually normal blastocysts were selected and transferred to recipient cows.

13. Genomes of recipient cows, nuclear donor cells, and cloned calves were typed for microsatellites by means of 23 primer sets that were provided by Shirakawa Institute of Animal Genetics, Livestock Technology Association of Japan [M. M. Inoue *et al.*, *Anim. Sci. Technol.* **68**, 443 (1997)].
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Elevating the Vitamin E Content of Plants Through Metabolic Engineering

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α-Tocopherol (vitamin E) is a lipid-soluble antioxidant synthesized only by photosynthetic organisms. α-Tocopherol is an essential component of mammalian diets, and intakes in excess of the U.S. recommended daily allowance are correlated with decreased incidence of a number of degenerative human diseases. Plant oils, the main dietary source of tocopherols, typically contain α-tocopherol as a minor component and high levels of its biosynthetic precursor, γ-tocopherol. A genomics-based approach was used to clone the final enzyme in α-tocopherol synthesis, γ-tocopherol methyltransferase. Overexpression of γ-tocopherol methyltransferase in *Arabidopsis* seeds shifted oil compositions in favor of α-tocopherol. Similar increases in agricultural oil crops would increase vitamin E levels in the average U.S. diet.

The chloroplasts of higher plants produce numerous compounds that not only perform vital functions but also are important from agricultural and nutritional perspectives. Tocopherols, the lipid-soluble antioxidants known collectively as vitamin E, are one such group of compounds. The four naturally occurring tocopherols, α-, β-, γ- and δ-tocopherol, differ only in the number and position of methyl substituents on the aromatic ring (*1*). In addition to their role as antioxidants (*1*), tocopherols stabilize polyunsaturated fatty acids within lipid bilayers by protecting them from lipoxygenase attack (*2*).

Of tocopherol species present in foods, α-tocopherol is the most important to human health, has the highest vitamin E activity (*3*), and occurs as a single (*R,R,R*)-α-tocopherol isomer (*4*). Although all tocopherols are absorbed equally during digestion, only (*R,R,R*)-α-tocopherol is preferentially retained and distributed throughout the body (*5*).

The most recent U.S. recommended daily allowance (RDA) suggests that 10 to 13.4 international units (IU) of vitamin E [equal to 7 to 9 mg of (*R,R,R*)-α-tocopherol] be consumed daily (*6*). Because of the abundance of plant-derived components in most diets, this RDA is often met in the average diet. However, daily intake of vitamin E in excess of the RDA (100 to 1000 IU) is associated with decreased risk of cardiovascular disease and some cancers, improved immune function, and slowing of the progression of a number of degenerative human conditions (*5*). Obtaining these therapeutic levels of vitamin E from the average diet is nearly impossible unless a concerted effort is made to ingest large quantities of specific foods enriched in vitamin E.

In the United States, approximately 60% of dietary vitamin E intake is from vegetable oils (*7*). In soybean oil, which accounts for 80 and 25% of the edible oil consumed in the United States and the world, respectively (*8*), α-tocopherol and its immediate biosynthetic precursor γ-tocopherol account for 7 and 70%, respectively, of the total tocopherol pool (*9*). The other major oilseed crops—corn, canola, cottonseed, and palm oils—have similarly low α- to γ-tocopherol ratios (*4*).

Substantial increases in the α-tocopherol content of major food crops are needed to provide the public with dietary sources of vitamin E that can approach the desired therapeutic levels. The observation that many oilseeds contain relatively high levels of γ-tocopherol, the biosynthetic precursor to α-tocopherol, suggests that the final step of the α-tocopherol biosynthetic pathway, catalyzed by γ-tocopherol methyltransferase (γ-TMT), is limiting in these tissues. Therefore, it may be possible to convert the large pool of γ-tocopherol present in seeds such as soybeans to α-tocopherol by targeted overexpression of

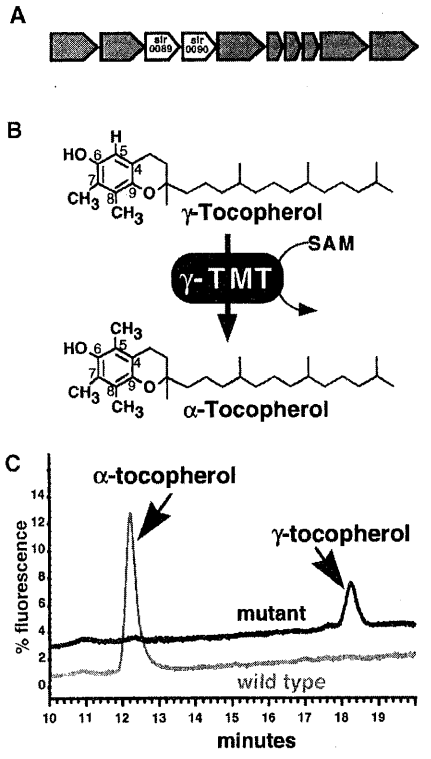

Fig. 1. γ-TMT in *Synechocystis* PCC6803. (**A**) Putative tocopherol biosynthetic operon from *Synechocystis* (*15*). SLR0089 encodes γ-TMT and SLR0090 encodes *p*-hydroxyphenylpyruvate dioxygenase. (**B**) γ-TMT enzymatic reaction. γ-TMT adds a methyl group to ring carbon 5 of γ-tocopherol. (**C**) HPLC profiles of tocopherols in wild-type *Synechocystis* PCC6803 and the γ-TMT null mutant. Total lipid extracts were isolated from each line, and tocopherols were analyzed by HPLC (*21*).

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