健康長寿の科学･文化を考える “フォーラム 10 年の歩みと未来への提言”
財団法人静岡総合研究機構･静岡学術フォーラム事務局 編，静岡県／静岡健康･長寿学術フォーラム組織委員会, pp. 91-99 (2006)

粘膜免疫と植物工学の融合：植物の改良と活用

静岡県立大学食品栄養科学部・21 世紀 COE プログラム　教授
小林 裕和

経歴
- 1982　名古屋大学 大学院農学研究科博士後期課程 農芸化学専攻 満了
- 1982　博士学位 (名古屋大学農学博士) 授与
- 1982　昭和 57 年度 日本学術振興会奨励研究員 (一般)　名古屋大学 農学部 生化学制御研究施設
- 1983　昭和 57 年度 日本学術振興会海外特別研究員　アメリカ合衆国 ハーバード大学 生物学教室
- 1984　名古屋大学 アイソトープ総合センター 助手
- 1991　静岡県立大学 大学院生活健康科学研究科 (食品栄養科学部) 助教授
- 1993　岡崎国立共同研究機構 基礎生物学研究所 客員助教授 (併任：1998 まで)
- 2003　静岡県立大学 大学院生活健康科学研究科 (食品栄養科学部) 教授

世界的な食糧危機の回避，機能性食品の生産，さらに創薬の観点から，遺伝子操作による植物の活用の意義は大きい．薬草として，植物は従来から医薬品の供給源であるが，植物遺伝子操作技術の発展とあいまって，遺伝子組換え植物を用いた医薬品生産はそのコストの低減化という観点から，実用的価値が極めて高い．たとえば，免疫グロブリンA (IgA) は，動物培養細胞を用いて生産すると 1 グラム当たり 10,000 米ドル近くかかるが，遺伝子組換え植物を用いると 100 米ドル以下に抑えられると試算される．「医薬分子農業」として，治療／診断のための抗体，ヒトや動物のためのワクチン，および健康に必要な生物薬剤が生産されようとしている．これらの医薬品は，純化の経費を考えると，注射より経口投与が前提とされるべきで，野菜や果物自身あるいは粗抽出物が用いられる．このように純化しないで用いるとすると，なおさら，食べて安全な遺伝子組換え植物を作出する技術の開発が望まれる．

現在までに遺伝子組換え農作物はどれほど普及しているのであろうか？　厚生労働省によって輸入が認可されている遺伝子組換え農作物は，現時点で 61 品目に及び，現在でも害虫抵抗性と除草剤耐性農作物は代表的なものである．しかしながら，国内では未だ販売を目的とした遺伝子組換え農作物の栽培は行われていない．害虫抵抗性や除草剤耐性の遺伝子は，ともに細菌に由来する．これらの遺伝子に加えて，遺伝子組換え操作には，遺伝子組換え体を選抜するために，「選択マーカー」と呼ばれる遺伝子が不可欠である．選択マーカーとしては，抗生物質耐性遺伝子や除草剤耐性遺伝子が使われるが，これらもまた主たるものは細菌由来である．抗生物質耐性遺伝子は，薬剤耐性菌の出現につながる危険性もあり，特に歓迎されない．遺伝子組換え農作物の作出に必要な選択マーカーを本来食に供している植物などから提供すべきと考える．一方，遺伝子組換え農作物からの花粉を介した外来遺伝子の飛散が懸念され，これは，「遺伝子汚染」として問題視されている．植物の細胞には，太陽光を受けて酸素を発生し空気中の炭酸ガスから糖を生産する工場が存在し，これを「葉緑体」と呼ぶ．この葉緑体にある遺伝情報は，通常の植物では花粉に入らないため，葉緑体への遺伝子導入により，花粉を介した遺伝子汚染の危険性が回避できる．

安全な遺伝子組換え植物を作出する技術として，植物由来の (したがって安全な) 選択マーカーを用いた葉緑体遺伝子導入法 (したがって花粉を介した遺伝子汚染を伴わない) を開発している．この目的に対する選択マーカーとして，葉緑体で行われるアミノ酸合成の酵素に着目し，この酵素に各種アミノ酸置換を導入して，これらの改変酵素の除草剤耐性能を検討した．除草剤耐性型アミノ酸合成酵素の遺伝子を用いて，アブラナ科植物やタバコへの葉緑体遺伝子導入を試みている．本法により，市場に受け入れられやすい遺伝子組換え農作物や植物による医薬品の生産が可能になると考える．

Cooperation between Mucosal Immunology and Plant Biotechnology: Genetic Modification of Plants

Hirokazu Kobayashi

Professor, COE Program in the 21th Century,
School of Food and Nutritional Sciences, University of Shizuoka

---

Past Records	1982	Ph.D. (Nogaku-Hakushi), Plant Biochemistry, Nagoya University, Japan
	1982	Postdoctoral Fellow of JSPS, School of Agriculture, Nagoya University, Japan
	1983	JSPS Postdoctoral Fellow for Research Abroad, Biological Laboratories, Harvard University, USA
	1984	Assistant Professor, Radioisotope Research Center, Nagoya University, Japan
	1991	Associate Professor, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Japan
	1993	Associate Professor (Adjunct), Laboratory of Biological Regulation and Photobiology, National Institute for Basic Biology, Japan (until 1998)
	2003	Professor, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Japan

Introduction

The exhaustion of foods and aggravation of the environment worldwide, as well as the society aging, are well considered in a today's society. Those aspects lead us an advanced society harmonizing with nature aiming at healthy longevity of humans. Plants can utilize the solar energy and synthesize carbohydrates from atmospheric CO_2, providing the food resources to humans. The atmospheric CO_2 causes the greenhouse effect on the earth, and thus able to be absorbed by plants. Majority of pharmaceuticals and nutraceuticals in human health and longevity are delivered from plants including herbs. Therefore, the improvement of plants is the most significant aspect for the continuous prosperity of humans, and safety development of genetic manipulation of plans for both the environment and oral intake.

Genetic manipulation of plants is invaluable for possible evasion of a global food crisis, production of functional foods, and drug design and production. Plants have just met the technology of genetic manipulation, and are potential sources of production of medicines at lower costs. For example, to produce an immunoglobulin G (IgG) from transgenic alfalfa grown in a 250-m^2 greenhouse costs US$500-600 per gram which is less expensive comparing to US$5,000 per gram for the hybridoma-produced antibody (1). Planet Biotechnology (Mountain View, CA, USA) has compared the cost per gram of purified IgA made by cell culture, transgenic goats, grain (7.5 tonne ha^{-1}) and green biomass (120.0 tonne ha^{-1}). Expression levels indicates a significant cost impact: the best expression level has been reported that leaves contain 500 μg per gram of leaves for a secretory immunoglobulin A (IgA), and the final cost should be well below US$50 per gram (1). This drastically undercuts the costs of cell culture (US$1,000 g^{-1}) or that of transgenic animal production systems (US$100 g^{-1}) (1). The biggest cost component with "plantibodies" is purification. However, the seed expression for rice and wheat helps the possibility of oral administration of some therapeutic antibodies without expensive purification. As "medical molecular farming", several trials are being achieved for production of therapeutic and diagnostic antibodies, human or animal vaccines, and biopharmaceuticals for human health in transgenic plants. These plant products are rather suited for oral administration than injection in consideration of expense for their purification: vegetables or fruits themselves or their crude extracts should be orally taken by humans. Especially for taking them without purification, safe technology to make genetically-modified plants as being developed by us (see below) may meet consumers' acceptance.

Genetically-modified (GM) Crops

How is the current available condition of genetically-modified (GM) plants in public? Varieties of genetically-modified crops imported to Japan are authorized by Ministry of Health, Labour and Welfare (MHLW) of Japan: those include approximately 73 in species of potatoes, soybeans, sugar beets, corn plants, rape seeds, cotton, and alfalfa (on December 15, 2005; http://www.mhlw.go.jp/topics/idenshi/). Herbicide tolerance, pest resistance, disease resistance, higher production of

oleic acid, and male sterility-fertility control are genetically conferred on the GM plants. Currently, any of these crops have not yet been cultivated within Japan for commercial purposes, but they all are imported instead.

A genetic material for BT protein (BT) from the bacterium *Bacillus thuringiensis* governs the pest resistance. Although plants are transformed with BT gene which tends to have negligible impact on non-target organisms, BT corn plants might represent a risk because most hybrids express BT and corn pollen is dispersed over at least 60 meters by wind. Hypothetically corn pollen is spread to other plants near corn fields and this can be ingested by the non-target organisms that consume these plants. A laboratory assay found that larvae of the monarch butterfly, *Danaus plexippus*, reared on milkweed (*Asclepias curassavica*) leaves dusted with pollen from BT corn, ate less, grew much slower and suffered higher mortality than those on leaves dusted with untransformed corn pollen or leaves without pollen (2). In detail, pollen from N4640-BT corn and an unrelated, untransformed hybrid, was applied by gently tapping a spatula of pollen over milkweed leaves that had been lightly misted with water (2). Pollen density was set as visually match densities on milkweed leaves collected from corn fields. Petioles of individual leaves were placed in water-filled tubes which were taped into plastic boxes. Five three-day-old monarch larvae from their captive colony were placed on each leaf, and each treatment was replicated five times. Milkweed leaf consumption, monarch larval survival and final larval weight were recorded over four days. Larval survival (56%) after four days of feeding on leaves dusted with BT pollen was significantly lower than that on leaves dusted with untransformed pollen or control leaves with no pollen (2). Because of no mortality on leaves dusted with untransformed pollen, all of the mortality on leaves dusted with BT pollen can be concluded to be affected by BT. The description fact in this scientific correspondence published in May, 1999, came to light that movement for boycott to genetically-modified crops begun in Europe and then in Japan. As a result, as of April, 2001, the indication of GM crops was enacted in a government law in Japan. There are, however, blind spots in the law: contamination less than 5% with GM crops in their population does not require the indication, and the law is also inapplicable to materials such as oil and soy source manufactured from GM crops.

Genetic Materials in Plant Cells

The major genetic information for 25,000 or more genes is located in nuclei in plant cells (3), and genes a little over 100 are present in genomes of plastids such as chloroplasts (4) and less than 60 are identified in mitochondrial ones (5). To deliver genetic information into nuclei and chloroplasts, biological, physical, and chemical developments are required, whereas no stable transformation of mitochondrial genomes has been reported so far. For nuclear genomes, *Agrobacterium* Ti plasmid mediation, particle bombardment, polyethylene glycol (PEG) treatment, and electroporation are available, and for chloroplast genomes, particle bombardment and PEG treatment are procedures developed (Fig. 1). Commercially available GM crops are consisted in manipulation of nuclear genomes, which is easer than that of chloroplast genomes whose copy number is over a few thousands per cell, resulting in difficulty in complete replacement with manipulated species. The advantages of nuclear engineering are easiness and capability of glycosylating proteins in cytoplasm (Golgi apparatus). The most useful character in chloroplast transformation is maternal inheritance, which resists “genetic pollution” via pollen scattering in the fields. Furthermore, chloroplast transformation is associated with the following benefits: homologous recombination leading to efficient DNA replacement, higher gene expression, polycistronic transcription for expression of multiple genes, accumulation of products such as proteins and metabolites, and improvement of photosynthesis (Table 1).

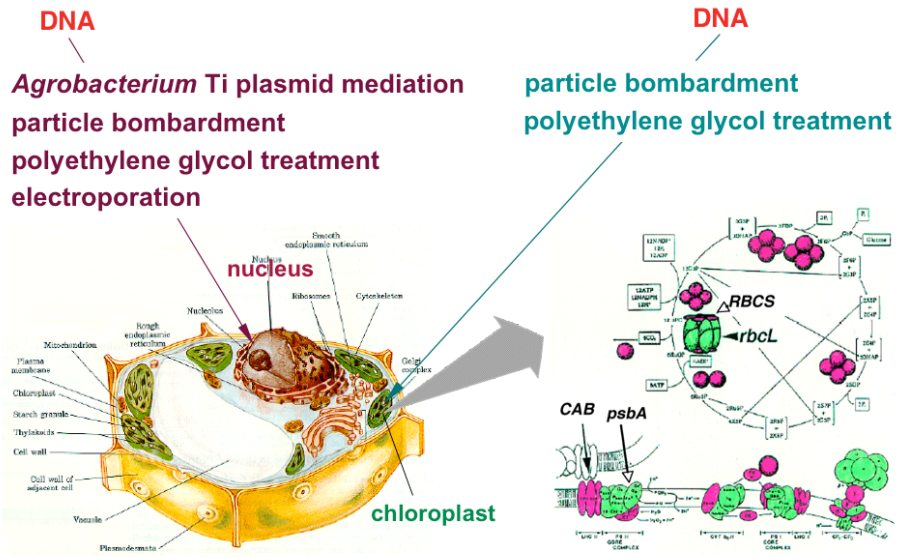

Fig. 1. Ways of gene delivery into nuclear and chloroplast genomes in plant cells.

Table 1. Advantages in transformation of two distinct genomes in plant cells: nuclei vs chloroplasts.

	Nuclei	Chloroplasts
Easiness	++	-
Homologous recombination (DNA replacement)	+	++
Higher gene expression	+	++
Polycistronic transcription	-	++
Accumulation of products	+	++
Maternal inheritance (No transfer of foreign genes via pollen)	-	++
Improvement of photosynthesis	+	++
Protein glycosylation	++	-

Chloroplast Transformation

Current protocols for plastid transformation employ strategies to obtain homoplastomic plants by segregating genome copies and organelles in somatic cells. The most common approach to plastid transformation in tobacco is introduction of foreign genes into chloroplast genomes in leaves and regeneration of shoots from transformed cells on a selective medium (6). Formation of homoplastomic cells is accelerated by chloroplast to proplastid dedifferentiation, with a concomitant reduction in plastid DNA number in tissue culture cells, then a rebuilding of the organelle and plastid DNA numbers in regenerated plants (6). Transplastomic shoots regenerated from leaves after bombardment are always chimeras. Spectinomycin and kanamycin resistance, conferred by the expression of chimeric genes, is neither cell-autonomous in regenerating shoots nor in seedling cotyledons. Lack of cell-autonomous expression means that, in chimeric shoots, nontransformed sectors also have a resistant (green)

phenotype, although they become bleached when cut out and placed in direct contact with the selective medium. Resistant phenotype of nontransformed cells in a chimeric plant is due to cross-protection by transformed cells. However, transformed and nontransformed sectors can be readily identified by color (green or white) in knockout plants lacking a photosynthetic gene or by green fluorescent protein (GFP) (7) accumulation, which are cell-autonomous traits (6). The preferred method to obtain homoplastomic tobacco plants is regenerating new shoots from the transplastomic sectors, which are then rooted. Homoplastomic plants from the chimeric shoots can also be obtained in the seed progeny, as long as the transplastomes are present in the cell layer that contributes to the maternal germline (6). Homoplastomic plants can be obtained directly from tissue culture cells if cells (protoplasts) are first cultured to form undifferentiated callus, and plant regeneration is delayed until plastid segregation is complete. However, extended propagation of cells in tissue culture is undesirable because it causes chromosome rearrangements and polyploidization that affect plant fertility (6).

Enhanced Expression in Chloroplasts

The chloroplast is a semi-autonomous organelle whose genetic information is encoded in the nuclear and plastid genomes. The plastid genome encodes genes for photosynthesis, as well as genes for housekeeping such as protein synthesis. There is the evidence that photosynthesis genes are transcribed by a multimeric *Escherichia coli*-type RNA polymerase (RNAP), and housekeeping genes are transcribed by a monomeric T7 or T3 bacteriophage-type RNAP (8). The *E. coli* RNAP is composed of a core complex of α, β, and β' subunits and one of a variety of s factors, the principal one being σ^{70}. Genes for s-like factors of *E. coli*-type RNAP had not been characterized from any multicellular eukaryotes, although they likely played a crucial role in the expression of plastid photosynthesis genes. We have cloned finally 6 distinct cDNAs designated *SIG1* to *SIG6* for polypeptides possessing amino acid sequences for domains conserved in σ^{70} factors of bacterial RNAP from the higher plant *Arabidopsis thaliana* (8). Each gene is represented as one copy per haploid genome without any additional sequences hybridized in the genome. Transient expression assays using GFP (7) demonstrated that N-terminal regions of the *SIG* open reading frames could function as transit peptides for import into chloroplasts. Transcripts for all *SIG* genes were detected in leaves but not in roots, and induced in leaves of dark-adapted plants in rapid response to light illumination (8). Together with results of our previous analysis of tissue-specific regulation of transcription of plastid photosynthesis genes, expressed levels of the genes may influence transcription by regulating RNAP activity in a green tissue-specific manner. The enhanced expression of *SIG* genes, as well as regulation of phosphorylation of SIGs, may promise to accumulate foreign gene products, proteins themselves and enzymes involved in metabolite production in chloroplasts, with establishment of chloroplast gene manipulation.

The higher expression in an ideally managed manner of endogenous and foreign genes in the chloroplast is desired for photosynthetic productivity enhancement and efficient production of modified substances therein. However, strength of gene promoters for potentially higher expression of recombinant genes in the chloroplast had not intensively been compared under the common experimental condition. We have focused on possible stronger promoters in the chloroplast: those of *psbA* encoding D1 protein of photosystem II reaction center, 16S rDNA in *rrn* operon, the bacterial fused promoter *tac*, and the bacteriophage T7 gene *ϕ10* in combination with transgenic T7 RNAP. *Arabidopsis* plants were made transgenic in the nuclear genome with the construct of a chimeric gene for T7 RNAP fused to a chloroplast transit peptide at its N-terminus placed under the control of CaMV 35S promoter (9). We have transiently expressed gene for β-glucuronidase (GUS) under control of the above promoters in the *Arabidopsis* chloroplast followed by particle bombardment. Expression in the chloroplast but not in the nucleus was confirmed histochemically and by a-amanitin treatment, the inhibitor of nuclear RNAP II. T7 promoter was the strongest in the examined promoters in the *Arabidopsis* chloroplast, and it is applicable to higher expression of foreign genes in the chloroplast with managed expression of T7 RNAP (9).

Selectable Marker Genes

There are crops on which pest resistance or herbicide tolerance is genetically conferred as mentioned above. Employed genes for these properties are derived from bacteria which are not eaten by humans. In addition to these genes, the genes called "selectable markers" are required for production of genetically-modified plants. The selectable markers used for selection of plants in which objective genes are successfully integrated into genomes of host plants, are genes for tolerance to antibiotics or herbicides, the majority of which are derived from bacteria too: genes for neomycin phosphotransferase II (*nptII*) from *Escherichia coli* Tn5, 5-enoylpyruvate shikimate-3-phosphate synthase (*epsps)* from *Agrobacterium* sp. CP4, phosphinothricine acetyltranasferase (*pat)* from *Streptomyces viridochromogenes*, aminoglycoside-3"-adenyltransferase (*aadA*) for spectinomycin resistance from *Shigella flexneri*, *etc*. (10). The safety of especially genes for antibiotic resistance is suspicious, because of the possibility that these genes might be transferred into pathogenic bacteria which may convert to antibiotic-resistant ones. Search of the selectable markers from plants is desired.

A Safer Plant-origin Selectable Marker

The present society requires safety in the "public acceptability" for both oral intake and the environment. What is the criterion of the public acceptability? The safety of all MG crops imported into Japan is authorized by MHLW on the basis of experimental proof. However, consumers judge the crops in likes or dislikes. People do not want to eat crops are genetically modified with genes of bacterial origin. Therefore, selectable marker genes are ideally to be plant origin. The safety in the environment, another important factor which we must consider, is to minimize so called "genetic pollution" of influence on the ecosystem. Transfer of foreign genes into other non-transgenic plants is most reliable via pollen. This apprehension is dispelled by chloroplast transformation. Since genes in plastids in most plant species are inherited maternally, being those genes not transferred into other plants via pollen. Therefore, the development of genetic manipulation of plastid genomes, instead of nuclear ones whose engineering has been established, is necessary for ecological safety.

To satisfy both plant origin and applicability for chloroplast transformation, we have focused on an enzyme involving amino acid biosynthesis in plastids, acetolactate synthase (ALS) [acetohydroxyacid synthase (AHAS)]. A variety of amino acid substitutions have been introduced into this enzyme and its tolerance to herbicides has been evaluated (patents pending, in collaboration with Kumiai Chemical Industry Co., Ltd., Tokyo). We have made constructs to express them in plastids in brassica plants and tobacco. This marker has proven to also function when it is expressed in nuclei (unpublished), being applicable for IgA and IgG production in plants, which are glycoproteins.

Validity of "Greened" Culture Cells

The success in application of our developed technology in a shorter period is expected in addition to longer-term projects, and utilization of cultured plant cells for production of pharmaceuticals and nutraceuticals would be more rapidly applied than the establishment of new plant cultivars genetically modified. Plant cells are usually cultured under heterotrophic conditions in supplementation with sugar, resulting in nongreen cells propagating. However, plant cells are vital with production of a variety of substrates by function of green chloroplasts. We have succeeded in isolating a few genes for "greening" via stimulation of biogenesis of chloroplasts.

In an effort to identify "greening genes", *Arabidopsis* lines homozygous for each transgene construct made with the gene for hygromycin B phosphotransferase or GUS placed under control of the promoter of the nuclear gene for the small subunit of ribulose-1,5-bisphosphate carboxylase/ oxygenase so called "Rubisco" (*RBCS-3B*) were constructed (11). Furthermore, activation tagging with T-DNA possessing quadruply-repeated enhancers derived from the cauliflower mosaic virus 35S promoter was applied to the transgenic line of *Arabidopsis* (11). Mutants resistant to hygromycin B during the growth of calli generated from non-green roots on callus-inducing medium resulted from the expression of hygromycin B phosphotransferase driven by the *RBCS-3B* promoter. Three mutant lines, *ces101* to *ces103*

(*callus expression of RBCS*), were obtained from approx. 4,000 calli resistant to a selectable marker for transformation (Fig. 2). The active transcription driven by the *RBCS-3B* promoter in all the calli of *ces* mutants was confirmed by expression of both GUS reporter gene and endogenous *RBCS-3B*. Chlorophyll and carotenoids, as well as light-dependent O_2 evolution, have been detected in the calli of all *ces* mutants. The loci where T-DNA was integrated in *ces101* line were determined by thermal asymmetric interlaced (TAIL)-PCR. The introduction of a DNA fragment harboring gene for receptor-like kinase placed under the influence of enhancers into the parental line, reproduced the phenotype of *ces* mutants. We have thus concluded that CES101 is receptor-like kinase (11), and genes for CES102 and CES103 are applied for patents.

Plant Species Manipulated

There is significant interest in recombinant protein production in non-toxic, edible plant species not only to minimize downstream protein processing costs but also to develop a combined production and delivery system for "edible" protein therapies. Lettuce (*Lactuca sativa*) is a commercially important crop belonging to the Asteraceae. The leaves of this crop are consumed raw by humans and the time from sowing seed to edible biomass is only weeks compared to months for crops such as tomato or potato (12). Nuclear and chloroplast transformation of lettuce has been developed by Hiroshi Asao (Nara Agricultural Experimental Station, Kashihara, Nara), with whom we are collaborating to apply of our safer selectable marker to the transformation.

Even though our selectable marker is safer for plants orally taken in, the society, especially in Japan and Europe, is premature to accept manipulated plants with our marker. Consumers could currently prefer functional or medical components made by engineered plants but further purified. Shizuoka Prefecture is famous for tea production which contains polyphenols detoxifying active-oxygen species, the cause of cell aging and generation of cancer. Catechins, a group of polyphenols, are made by tea (*Camellia sinensis*) plants belonging to the family Theacea, and much consumed as additives of commercially-bottled tea in these years in Japan. Tea was described to be first taken in China as medicinal drink, later as beverage and have been doing so for the past 3,000 years, being the oldest non-alcoholic caffeine-containing beverage in the world. Tea is an evergreen, perennial, cross-pollinated plant and grows naturally as tall as 15 meters. However, under cultivated condition, the bush height of 60-100 cm is maintained for harvesting the tender leaves, which continues even more than 100 years (13). Although it is less easy to culture this plant *in vitro*, cultered cells of tea which are able to be regenerated have been established by Michiyo Kato (Numazu National College of Technology, Numazu, Shizuoka), who has provided us the cells and is collaborating with us for genetic transformation of tea to improve catechin productivity.

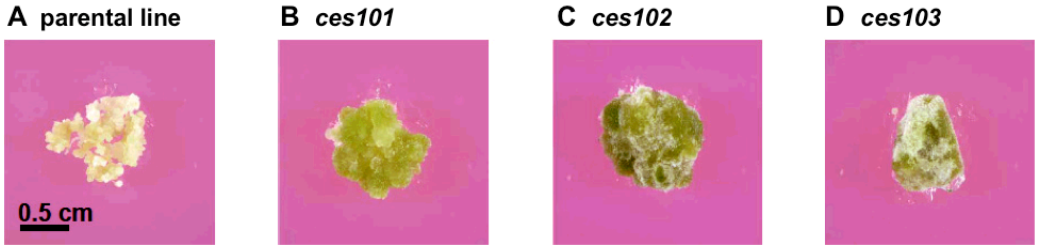

Fig. 2. "Greening genes" conferring chloroplast function to generate a verity of substrates on plastids in heterotrophically-cultured cells. Cultured cells derived from *Arabidopsis* roots exhibited green due to expression of the genes (11).

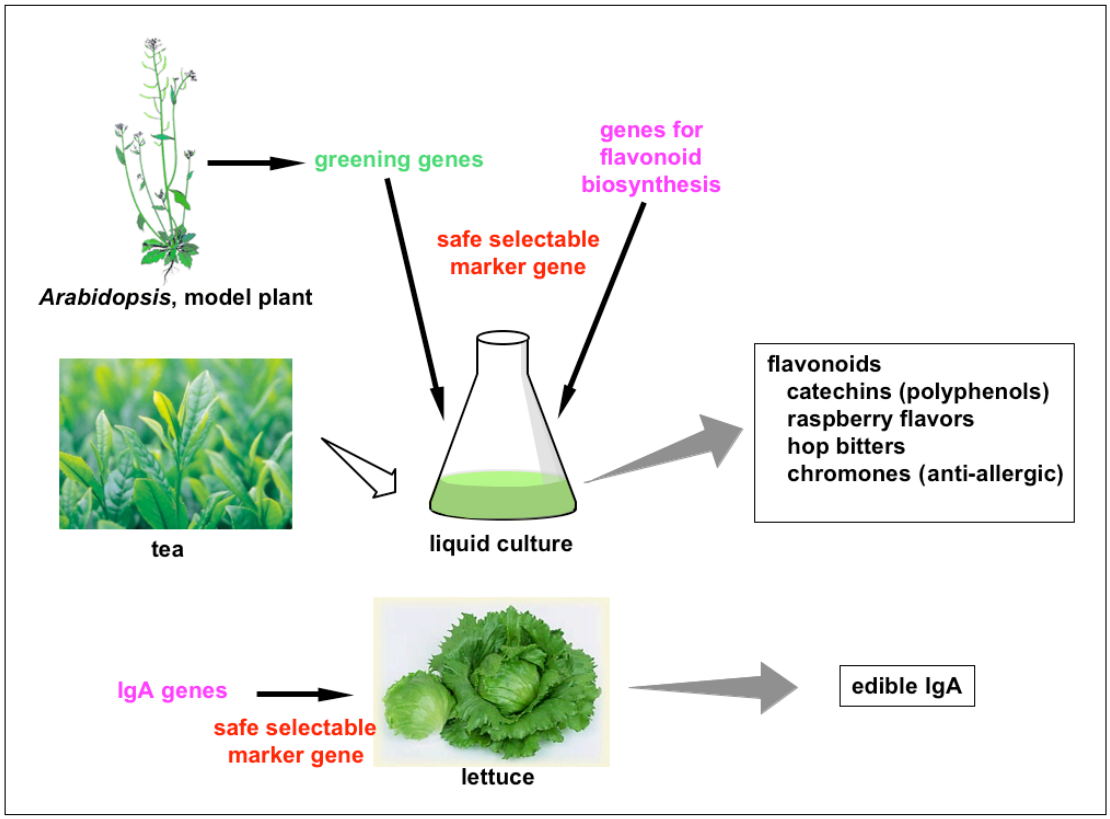

Fig. 3. Schematic representation of strategies for molecular farming for production of pharmaceutical and nutraceutical compounds. "Greening genes" discovered with the model plant *Arabidopsis* and genes for flavonoid biosynthesis are introduced into cultured tea cells by our original selectable marker gene, resulting in production of functional and medical compounds therein. The selectable marker gene is also employed for generation of lettuce which protects humans against pathogens by being eaten.

Perspectives

We put our knowledge and developed experimental systems together to produce pharmaceutical and nutraceutical compounds in plants. The most striking methodology is the employment of novel safer selectable marker of plant origin, which is used for both nuclear and chloroplast transformation; the former is necessary for glycoprotein production, and the latter is the safest in point of no scattering of foreign genes via pollen in the fields. We have also the knowledge of transcriptional regulation through s factors and the strongest promoters in chloroplasts, where foreign genes are expressed. We have initiated experiments with lettuce and tea cultured cells. The production of catechins (polyphenols) in tea cells in which genes for flavonoid biosynthesis are introduced by our selectable marker is in progress. Raspberry flavors and hop bitters as functional compounds, and anti-allergic chromones as medicines are planned to be produced in other plant systems (Fig. 3). Furthermore, lettuce accumulating IgA against microbial infection is being generated in collaboration with Yasuyuki Imai (School of Pharmaceutical Sciences, University of Shzuoka) (Fig. 3).

Plant-origin pharmaceuticals and nutraceuticals are orally taken in as vegitables themselves, juice made of cultured cells, or additives after purification. Edible MG plants for healthy longvity of humans as descrived in this article will be avialble and accepted by consumers hopfully in a few years.

References

1. Daniell, H., Streatfield, S.J. and Wycoff, K. (2001) Medical molecular farming: Production

of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci. 6, 219-226.
2. Losey, J.E., Rayor, L.S. and Carter, M.E. (1999) Transgenic pollen harms monarch larvae. Nature 399, 214.
3. The *Arabidopsis* Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408, 796-815.
4. Sato, S., Nakamura, Y., Kaneko, T., Asamizu, E. and Tabata, S. (1999) Complete structure of the chloroplast genome of *Arabidopsis thaliana*. DNA Res. 6, 283-90.
5. Unseld, M., Marienfeld, J.R., Brandt, P. and Brennicke, A. (1997) The mitochondrial genome of *Arabidopsis thaliana* contains 57 genes in 366,924 nucleotides. Nature Genet. 15, 57-61.
6. Maliga, P. (2004) Plastid transformation in higher plants. Annu. Rev. Plant Biol. 55, 289–313.
7. Niwa, Y., Hirano, T., Yoshimoto, K., Shimizu, M., and Kobayashi, H. (1999) Non-invasive quantitative detection and applications of nontoxic-, S65T-type green fluorescent protein in living plants. Plant J. 18, 455-463.
8. Isono, K., Shimizu, M., Yoshimoto, K., Niwa, Y., Satoh, K., Yokota, A. and Kobayashi, H. (1997) Leaf-specifically expressed genes for polypeptides destined for chloroplasts with domains of σ^{70} factors of bacterial RNA polymerases in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 94, 14948-14953.
9. Yoshimoto, K., Sakaiya, M., Isono, K. and Kobayashi, H. (2001) Comparison of strength of endogenous and exogenous gene promoters in *Arabidopsis* chloroplasts. Plant Biotechnol. 18, 135-142.
10. Hare P.D. and Chua N.-H. (2002) Excision of selectable marker genes from transgenic plants. Nature Biotechnol. 20, 575-580
11. Niwa, Y., Goto, S., Nakano, T., Sakaiya, M., Hirano, T., Tsukaya, H., Komeda, Y. and Kobayashi, H. (2006) *Arabidopsis* mutants by activation tagging in which photosynthesis genes are expressed in dedifferentiated calli. Plant Cell Physiol. 47, 319-331.
12. Lelivelt, C.L., McCabe, M.S., Newell, C.A., Desnoo, C.B., van Dun, K.M., Birch-Machin, I., Gray, J.C., Mills, K.H. and Nugent, J.M. (2005) Stable plastid transformation in lettuce (*Lactuca sativa* L.). Plant Mol Biol. 58, 763-774.
13. Mondal, T.K., Bhattacharya, A., Laxmikumaran, M. and Ahuja, P.S. (2004) Recent advances of tea (*Camellia sinensis*) biotechnology. Plant Cell, Tissue Organ Culture 76, 195–254.