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Molecular Farming with Cultured Cells

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Introduction

The Industrial Revolution of the late 18th century contributed to the current prosperity of mankind and an increased longevity in humans, with the average life span of males and females in Japan changing from 50 and 54 years in 1947 to the present values of 79 and 85 years, respectively (1, 2). The longest life span is recorded for inhabitants of Japan, and contrasts with the short life expectancy of 32 years for residents of South Africa (2). The aged (65 years-old and over) population in Japan was around 4 million in 1947 and has now become 27 million, while the rate of increase has doubled in 20 years and is not expected to fall below this level in the following two decades (1). However, longevity is associated with serious problems concerning lifestyle-related illness, and the growing population of aged patients (3.2 million in 2005 in Japan) has led to higher medical care expenses that have reached 12 trillion yen per year in Japan (3).

Obesity, diabetes, hyperpiesia, lipemia, hyperuricemia and cancer are known as lifestyle-related illnesses. Malignant tumors, heart disease and cerebrovascular disease resulting from lifestyle-related illnesses are regarded as the 3 major causes of death, followed by pneumonia; these 4 diseases account for nearly 90% of the total causes of death (1, 3). Lifestyle-related illnesses are triggered by an inadequate intake of foods, which are ironically abundant in advanced countries such as Japan but insufficient in developing countries. To reduce the expense of medical care in a society, health and longevity are desired. The increased prevalence of lifestyle-related illness has led people to realize the importance of functional foods. Most pharmaceuticals and nutraceuticals necessary for preventing lifestyle-related illness and fostering longevity and health are derived from plants and herbs, to which serious attention is currently paid.

Genetic engineering of plants may improve plants to allow them to accumulate more functional compounds in a shorter period than by ordinary plant breeding, although this technology is not accepted by consumers and boycotts of genetically modified (GM) crops began in Europe in 1999 and then appeared in Japan. This movement is unavoidable and GM plants must be carefully evaluated from two aspects: safety with respect to direct oral intake, and their impact on the ecological system through so-called “genetic pollution”. In this context, the utilization of cultured plant cells in closed systems for the production of pharmaceuticals and nutraceuticals, as well as the distribution of compounds extracted from such cells, would be more easily applied to a commercial system than the establishment of new GM plant cultivars for cultivation in fields.

Flavonoids as Major Pharmaceuticals and Nutraceuticals

The flavonoids, with more than 4,500 known representatives, constitute an enormous class of phenolic natural products (4). Present in most plant tissues and often in vacuoles, flavonoids can occur as monomers, diners and higher oligomers, mostly as glyosides. They are also found as mixtures of colored oligomeric/polymeric components in various heartwoods and barks. Various flavonoids have also been studied extensively from the perspectives of health protection and pharmacological utility, for which mammalian enzyme systems have been used to assess flavonoid activity. Flavonoids have been analyzed as modulators of immune and inflammatory responses, for their impact on smooth muscle function, and as anticancer, antiviral, antitoxic and hepatoprotective

agents. There is considerable current interest in the use of isoflavonoids in cancer prevention. Dietary consumption of the isoflavonoids daidzein and genistein, which are present in soybeans, is thought to substantially reduce the incidence of breast and prostate cancers in humans (4).

We are collaborating with Hiroshi Noguchi (Graduate School of Pharmaceutical Sciences, University of Shizuoka) to investigate flavonoids and their derivatives, such as catechins for cancerocidal and anti-oxidant effects, raspberry ketones for anti-obesity, chromones for anti-allergic function, curcumin for hepatic improvable and carcinostatic actions, and lindleyin as anti-inflammatory agents (Fig. 1) because we have cloned genes for enzymes involved with these flavonoids. In the biosynthesis of flavonoids, chalcone synthase (CHS) catalyzing the condensation of malonyl-CoA and *p*-coumaryl-CoA is a key enzyme. *p*-coumaryl-CoA is derived from phenylalanine, the precursors of which are synthesized in chloroplasts in green plant cells (Fig. 1).

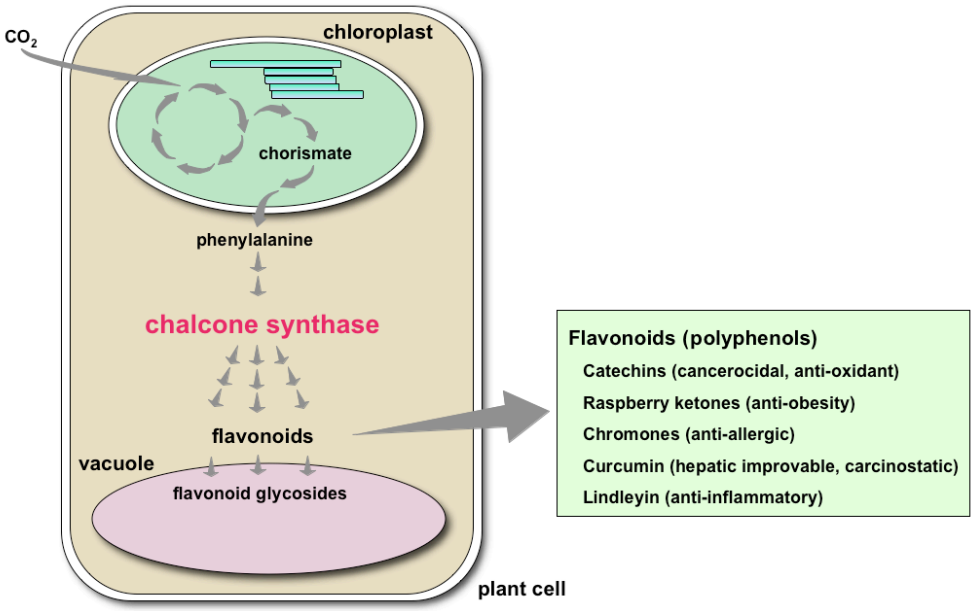

Fig. 1. Schematic representation of metabolic engineering for the production of pharmaceuticals and nutraceuticals with plant cultured cells.

Chloroplasts as Intracellular Factories

The chloroplast present in plant cells is one form of "plastid" that is plastic, a word that is derived from the Greek word "*plastis*", meaning a molder. Morphologically undifferentiated plastids are called proplastids. Differentiated plastids include chloroplasts possessing chlorophylls for photosynthesis, etioplasts in angiosperms grown without light, chromoplasts, which accumulate carotenoids, especially lycopene, in flowers and fruits, and leucoplasts such as amyloplasts, which are colorless and enriched with starch, being present in storage tissues including endosperm, cotyledons, root caps and tubers, as well as cultured plant cells (5).

The chloroplast is an intracellular factory where major substrates such as sugars, amino acids, lipids, terpenoids, carotenoids and porphyrins—all necessary for cellular functions—seem to be produced, in addition to being the site of photosynthetic activity through the utilization of solar energy. Plant cells may be vital for the production of such a variety of substrates by functioning green chloroplasts, where the shikimate pathway, for instance, may be crucial for the production of pharmaceuticals and nutraceuticals such as flavonoids via phenylalanine. However, in spite of the characterization of individual enzymes

and the prediction of transit sequences for the destination of proteins to plastids, inclusive analysis of functional proteins within chloroplasts is limited, as revealed by the present identification of 690 species of protein molecules as a champion data set (6).

We have isolated intact chloroplasts from the model plant *Arabidopsis thaliana* as free as possible from components in other intracellular compartments by repeating Percoll gradient centrifugation 3 times, with possible contamination with catalase as a marker enzyme for peroxisomes or fumarase for mitochondria less than 0.040% and 0.007%, respectively. The mixture of trypsin-digests of chloroplast proteins was subjected to novel two-dimensional HPLC/MS/MS [MudPIT (Multidimensional Protein Identification Technology)] (7). We have demonstrated the importance of chloroplasts for the supply of many substrates through the occurrence of enzymes involved in more pathways of secondary metabolism than expected—approx. 1,600 protein species have been identified in green chloroplasts from *Arabidopsis* (7). Surprisingly, most enzymes responsible for pathways whose operation has been predicted have been successfully identified. These have included the major enzymes engaged in the shikimate pathway for synthesis of phenylalanine (Fig. 2).

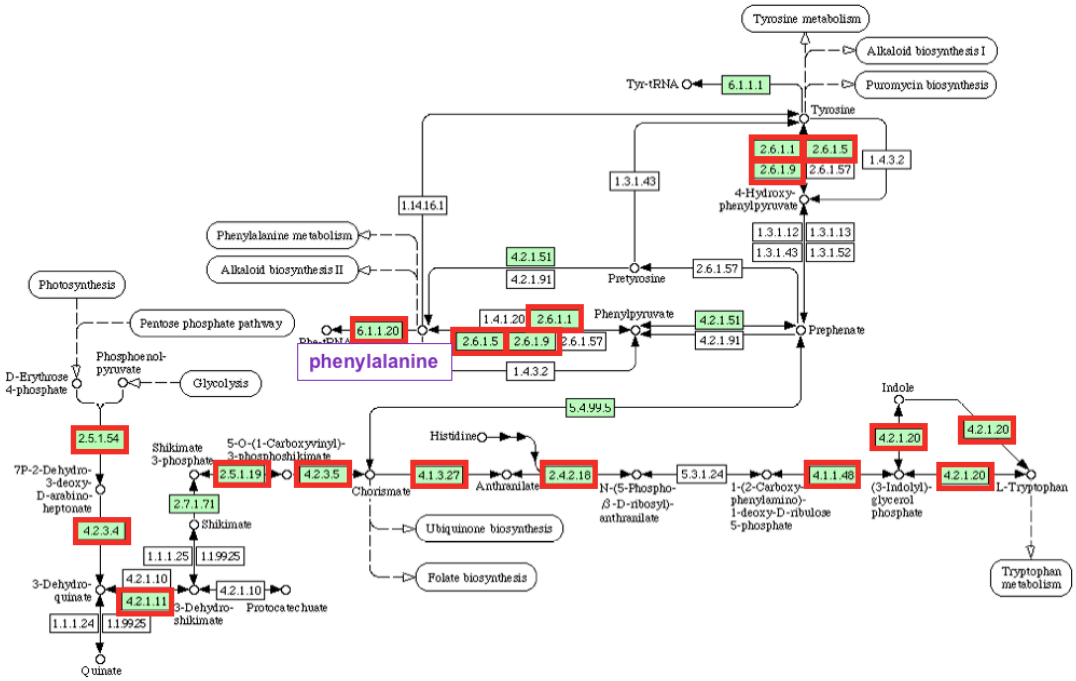

Fig. 2. Most of the enzymes engaged in the shikimate pathway of the chloroplast. *Arabidopsis* chloroplasts free from other contaminants (using Percoll gradient centrifugation, repeated 3 times) were subjected to novel two-dimensional HPLC/MS/MS [MudPIT (Multidimensional Protein Identification Technology)] after trypsin digestion. Detected enzymes are enclosed with red rectangles.

"Greening Genes" for the Functional Chloroplasts

The success in application of our developed technology to commercialization in a shorter period is expected, in addition to longer-term projects, and utilization of cultured plant cells for the production of pharmaceuticals and nutraceuticals would be more rapidly applied than the establishment of new plant cultivars that have been genetically modified. Plant cells are usually cultured under heterotrophic conditions involving supplementation with sugar, resulting in the propagation of non-green cells. However, plant cells are vital for the production of a variety of substrates through the functioning of green chloroplasts. We have succeeded in isolating a gene for "greening" to promote the 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 were constructed and 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*) (8). 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* (8). 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. The active transcription driven by the *RBCS-3B* promoter in all the calli of *ces* mutants was confirmed by expression of both the 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 the *ces101* line were determined by thermal asymmetric interlaced (TAIL)-PCR. The introduction of a DNA fragment harboring a 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 a receptor-like kinase (8), and patents have been applied for the genes CES102 and CES103. The function of chloroplasts in supplying a variety of substrates for metabolism can be switched on by the activity of CES101 (Fig. 3).

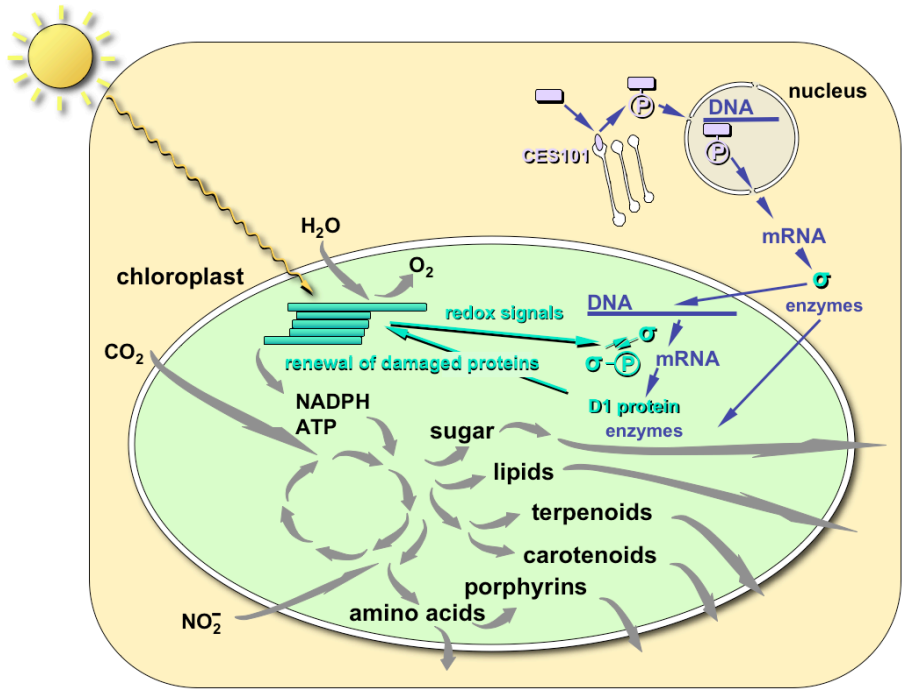

Fig. 3. Schematic representation of the functions of the chloroplast in supplying a variety of substrates for metabolism.

Manipulation of Plant Genes

What is the current state of GM plants in the public arena? Varieties of GM crops imported to Japan are authorized by the Ministry of Health, Labour and Welfare (MHLW) of Japan, and include approximately 76 cultivars comprising species of potato, soybean, sugar beet, corn plant, rapeseed, cotton and alfalfa (on August 15, 2006; 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 GM plants. None of these crops have been cultivated within

Japan for commercial purposes, and all are imported.

A genetic material for BT protein (BT) from the bacterium *Bacillus thuringiensis* determines pest resistance. Although plants transformed with the BT gene tend to have a negligible impact on non-target organisms, BT corn plants might represent a risk because most hybrids express BT and corn pollen is dispersed over a distance of at least 60 meters by wind. Hypothetically, corn pollen is spread to other plants near corn fields and this can be ingested by 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 larvae reared on leaves dusted with untransformed corn pollen or leaves without pollen (9). 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 (9). Pollen density was set as visually matched densities on milkweed leaves collected from corn fields. Petioles of individual leaves were placed in water-filled tubes that were taped into plastic boxes. Five three-day-old monarch larvae from a 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 (9). Due to the absence of mortality on leaves dusted with untransformed pollen, the mortality on leaves dusted with BT pollen can be attributed to the effects of BT. The disclosure of this scientific study in May of 1999 prompted a movement for the boycott of genetically modified crops that began in Europe and later appeared in Japan. As a result, an indication of the GM status of a crop was required by government law in Japan in April of 2001. However, there are blind spots in the law: contamination less than 5% with GM crops in a population does not require the indication, and the law does not apply to materials such as oil and soy source manufactured from GM crops.

Engineering of the Chloroplast Genome

The major genetic information for 25,000 or more genes is located in nuclei in plant cells (10), with a little over 100 genes being present in the genomes of plastids such as chloroplasts (11) and less than 60 genes identified in mitochondrial genomes (12). Biological, physical and chemical techniques are required to deliver DNA as genetic information into nuclei and chloroplasts, 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, while particle bombardment and PEG treatment are procedures available for chloroplast genomes. Commercially available GM crops have resulted from a manipulation of nuclear genomes. This approach is easer than manipulating chloroplast genomes whose copy number is over a few thousand per cell, resulting in difficulty in complete replacement with the manipulated species. The advantages of nuclear engineering are ease of manipulation and the potential for glycosylating proteins in the cytoplasm (Golgi apparatus). The most useful character in chloroplast transformation is maternal inheritance, which resists "genetic pollution" via pollen scattering in fields. Furthermore, chloroplast transformation is associated with the following benefits: homologous recombination leading to efficient DNA replacement, higher gene expression, polycistronic transcription for the expression of multiple genes, accumulation of products such as proteins and metabolites, and improvement of photosynthesis.

Current protocols for plastid transformation employ strategies to obtain homoplastomic plants by segregating genome copies and organelles in somatic cells. The most common approach for plastid transformation in tobacco is the introduction of foreign genes into chloroplast genomes in leaves and the regeneration of shoots from transformed cells on a selective medium (13). Formation of homoplastomic cells is accelerated by the chloroplast and leads to proplastid dedifferentiation, with a concomitant reduction in plastid DNA number in tissue culture cells and then a rebuilding of the organelle and plastid DNA numbers in regenerated plants (13). Transplastomic shoots

regenerated from leaves after bombardment are always chimeras. Spectinomycin and kanamycin resistance, conferred by the expression of chimeric genes, is not cell-autonomous in regenerating shoots or seedling cotyledons. Lack of cell-autonomous expression means that, in chimeric shoots, non-transformed sectors also have a resistant (green) phenotype, although they become bleached when cut out and placed in direct contact with the selective medium. A resistant phenotype of non-transformed cells in a chimeric plant is due to cross-protection by transformed cells. However, transformed and non-transformed sectors can be readily identified by color (green or white) in knockout plants lacking a photosynthetic gene or by green fluorescent protein (GFP) (14) accumulation, which are cell-autonomous traits (13). The preferred method to obtain homoplastomic tobacco plants is to regenerate 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 (13). Homoplastomic plants can be obtained directly from tissue culture cells if cells (protoplasts) are first cultured to form an 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 (13).

Safer Technology for the Genetic Modification of Plants

There are crops on which pest resistance or herbicide tolerance is genetically conferred as mentioned above. The genes used in such cases are derived from bacteria that are not eaten by humans. In addition to these genes, genes called "selectable markers" are required for production of GM 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 also derived from bacteria, and include 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*, and aminoglycoside-3"-adenyltransferase (*aadA*) for spectinomycin resistance from *Shigella flexneri.* (15). The safety of genes for antibiotic resistance is questionable because of the possibility that these genes might be transferred into pathogenic bacteria that may convert to antibiotic-resistant strains. A search for selectable markers from plants is therefore desired.

Citizens demand the safety of GM products in the form of "public acceptability" for both oral intake and the environment. What is the criterion for public acceptability? The safety of all GM crops imported into Japan is authorized by MHLW on the basis of experimental proof. However, consumers judge crops on the basis of likes or dislikes. People do not want to eat crops that are genetically modified with genes of bacterial origin. Therefore, ideal selectable marker genes should be of plant origin. The safety for the environment, another important factor that we must consider, is to minimize so-called "genetic pollution" or detrimental effects on the ecosystem. The transfer of foreign genes into other non-transgenic plants is most reliably performed via pollen. Apprehension associated with this process is dispelled by considering chloroplast transformation. Since genes in plastids of most plant species are inherited maternally, they represent genes not transferred into other plants via pollen. Therefore, the development of genetic manipulation of plastid genomes, instead of nuclear genomes whose engineering has been established, is necessary for ecological safety.

To satisfy both the need for a plant origin of selectable marker genes and applicability to chloroplast transformation, we have focused on an enzyme involving amino acid biosynthesis in plastids, acetohydroxyacid synthase (AHAS) [acetolactate synthase (ALS)]. A variety of amino acid substitutions have been introduced into this enzyme and its tolerance to herbicides has been evaluated (Fig. 4) (16). We have made constructs to express them in the plastids of brassica plants and tobacco. This marker functions when it is expressed in nuclei, being applicable to glycoprotein production in plants.

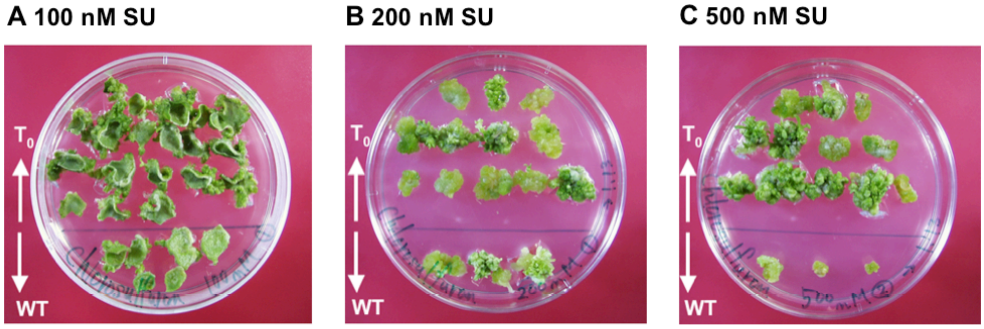

Fig. 4. Evaluation of the sustainable marker AHAS for chloroplast transformation of tobacco as an experimental model. AHAS was used for nuclear and chloroplast transformation.

Plant Species to be Cultured and Genetically Manipulated

Shizuoka Prefecture is famous for tea production, a plant product containing polyphenols that detoxify 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 are much consumed as additives of commercially bottled tea in Japan. Tea was first taken in China as a medicinal drink and later as a beverage; it is a foodstuff that has been consumed for the past 3,000 years and is the oldest non-alcoholic caffeine-containing beverage in the world. Tea is an evergreen, perennial, cross-pollinated plant and grows naturally to a height of up to 15 meters. However, under cultivated conditions, a bush height of 60–100 cm is maintained to allow harvesting of the tender leaves, a practice that has continued for more than 100 years (17). Although it is not easy to culture this plant *in vitro*, cultured cells of tea that can be regenerated have been established by Michiyo Kato (Numazu National College of Technology, Numazu, Shizuoka), who has provided us with such cells and is collaborating with us for the genetic transformation of tea to improve catechin productivity. We are also developing the tissue culture of other plant species to establish systems with high rates of cell propagation. It is worth paying attention to the roots of carrot (*Daucus carota*) and the purple sweet potato (*Ipomoea batatas*)—the former accumulate carotenoids and the latter is enriched with anthocyanin—cultured cells of which are genetically transformable and grow faster than those of tea.

Present-day societies, particularly those of Japan and Europe, would not accept manipulated plants with our marker, even though our selectable marker is safer. Consumers could prefer functional or medical components made by engineered plants, as long as they were further purified. There is significant interest for the production of recombinant enzymes and functional proteins in non-toxic edible plant species, not only to minimize downstream product processing costs but also to develop a combined production and delivery system for “edible” compounds and protein therapies. Lettuce (*Lactuca sativa*) is a commercially important vegetable belonging to the Asteraceae. The leaves of this plant are consumed raw by humans and the time from sowing the seed to producing edible biomass is only a few weeks, compared to months for crops such as tomato or potato (18). Nuclear and chloroplast transformation of lettuce has been developed by Hiroshi Asao (Nara Agricultural Experimental Station, Kashihara, Nara) (19), with whom we are collaborating to apply our safer selectable marker for the transformation.

Perspectives

We collaborated with other research groups and pooled our knowledge to develop experimental systems to produce pharmaceutical and nutraceutical compounds in plants. The most striking methodology is the employment of a 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 because there in no scattering of foreign genes via pollen in fields. We have also made advances concerning transcriptional regulation through σ factors and the strongest promoters in chloroplasts (20), where foreign genes are expressed. We have initiated work with tea cultured cells, and the *in vitro* culture of carrot and purple sweet potato. The production of catechins and other flavonoids in cultured cells in which genes for their biosynthesis are introduced by our selectable marker is in progress.

Pharmaceuticals and nutraceuticals of plant origin are consumed orally as vegetables themselves or through juice made from cultured cells. Edible GM plants that promote health and longevity in humans will hopefully be accepted by consumers following the commercialization of products extacted from cultured plant cells described in this article.

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