Characterization of Plant Functions Using Cultured Plant Cells, and Biotechnological Applications

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Plant cell cultures are widely used in the micro-propagation of clonal plants, especially virus-free plants, and in the production of useful metabolites such as paclitaxel. On the other hand, the use of plant cell cultures for the more basic characterization of plant functions is rather limited due to the difficulties associated with functional differentiation in cell cultures. In this review, I overview our experience with functionally differentiated cultured plant cells and their characteristics, especially with regard to photo-autotrophism and secondary metabolism. I emphasize the high potential of functionally differentiated cell cultures, as well as some of the pitfalls, in the characterization of plant functions and biotechnological applications.

Key words: benzylisoquinoline alkaloid biosynthesis; functional differentiation; photoautotrophic culture; secondary metabolism; somaclonal variation

Plant cell culture techniques are used in the micro-propagation of clonal plants, especially ornamental plants such as orchids and virus-free plants of crops propagated vegetatively. They are also used as a platform to establish genetic transformation and in the production of useful low-molecular-weight metabolites such as shikonin and paclitaxel. On the other hand, the use of plant cell cultures in the basic characterization of plant functions is rather limited due to the poor differentiation of cellular functions in cultured cells. Only a limited number of plant cell cultures, including tobacco BY2 cells, are used to characterize plant biochemistry and the physiology of basic processes in the cell cycle and cytoskeleton formation.

While it can be difficult to establish stably differentiated plant cell cultures due to their totipotency and concomitant high elasticity, they can provide greater flexibility in the analysis of plant functions in vitro and in biotechnological applications. Two examples of functionally differentiated cultured plant cells, photoautotrophic tobacco cells and cultured medicinal plant (e.g., Coptis japonica and Eschscholzia californica) cells that produce benzylisoquinoline alkaloids, are discussed below.

I. Establishment of Photoautotrophically Cultured Plant Cells and Their Application

1. Establishment of photoautotrophic (PA) cells

Plants can photosynthesize and grow without the addition of any organic carbon source. However, once plant tissues or cells are isolated from intact plants, they lose this photosynthetic ability, partially due to the effect of the sugars and plant growth hormones in the culture medium. In the establishment of a PA culture, cellular selection of green tissues using cellular variation (somaclonal variation) in the cultured cell population and subsequent evaluation of photoautotrophic properties with CO₂ enrichment are critical processes. Whereas cultured PA cells have been found to have lower chlorophyll contents than leaf mesophyll cells, the activities of photosystems I and II in PA cells are about half of those in leaf cells on a chlorophyll basis. PA cells have also been found to have relatively constant photosynthetic activity during the growth period, whereas photosynthetic activities in leaf cells change considerably with development. A growing body of evidence indicates that PA cells can be useful in the characterization of the photoautotrophic functions of leaf mesophyll cells, including photosynthetic signaling, photosynthesis-related metabolism, environmental responses, and photoautotrophism in vitro. While the PA cell cultures that have been established to date are largely limited to C3 photosynthetic dicotyledonous plant species, the recent identification of the chloroplast-development regulatory gene (GLK) has made possible the establishment of green cell cultures even from monocotyledonous plants. The establishment of monocot green cell cultures should lead to improved functioning of chloroplasts in monocots by means of cellular selection and genetic engineering through chloroplast transformation.

2. Applications of photoautotrophic (PA) cells

Once green PA cells have been established, they can be used to evaluate the effects of chemicals, including potential herbicides, photoautotrophic growth stimulators (e.g., cholines), and environmental stresses (e.g., NaCl) on chloroplast functioning (Fig. 2A). Green PA cells offer clear advantages over non-PA cells, since photosynthesis as a metabolic process is sensitive...
to various chemicals and stresses. For example, they can be used in the selection of herbicide-resistant plant cells and stress-tolerant cells, such as atrazine-resistant and NaCl-tolerant cells.5,11,13,14) The clear advantages of cell culture systems in which cells can be repeatedly exposed to sub-toxic levels of chemicals or environmental stresses have made it possible to isolate difficult mutants, even that of the chloroplast genome.5,13,14) The isolated chloroplast psbA mutant has yielded insight into the molecular mechanism of cross-resistance to

Fig. 1. Establishment of Photoautotrophically Cultured Tobacco Cells and Chloroplast Development.

Heterotrophically cultured cells, which are usually cultured in the dark, show poor development of chloroplasts. After cellular selection of highly green cells, a photomixotrophic cell culture was established, but these cells still showed poor development of chloroplasts, and CO$_2$ enrichment using a two-tier flask, as shown, was often needed to support the photoautotrophic culture. The bar in the electron micrographs is 1 µm. Abbreviations in the electron micrographs are as follows: C, chloroplast; CW, cell wall; M, mitochondrion; PG, plastoglobule; S, starch. The electron micrographs were adapted from ref. 28.

Fig. 2. Applications of Photoautotrophic Cells.

A, Photoautotrophic (PA) cultured cells were highly sensitive to herbicides as seedlings in comparison to photomixotrophic (PM) and heterotrophic (H) cells of tobacco.5) Arrows indicate the response of H cells to photosynthesis-inhibiting herbicides. B, 2-Dimensional-polyacrylamide gel electrophoresis analysis of total proteins in PA cells.16) N-Terminal amino acid sequencing of the polypeptides indicated that P1, P2, P4/P7, P15 and P17 were osmotin, chitinase, osmotin-like protein, small subunit of ribulose 1,5-bisphosphate carboxylase, and PsbP, respectively. C, Antifungal activity of purified PR-5.d.18)
diuron and atrazine in the D1 protein (a psbA gene product). These selected cells are clearly useful in further characterization of the photosynthetic apparatus, as discussed below.

3. Characterization of gene expression in cultured plant cells revealed that they were under stress

Comparative characterization of total protein profiles in cultured PA cells clearly indicated that they were considerably different from those in leaf mesophyll cells. The PA cells as well as other heterotrophically and photomixotrophically cultured cells accumulated large quantities of pathogenesis-related proteins (Fig. 2B), suggesting that they were under physiological stress. Hence, cultured plant cells should be used carefully in physiological characterization, but high accumulation of pathogenesis-related (PR) proteins in cultured cells provides a unique opportunity to study pathogenesis responses and PR-proteins under pathogen-free conditions. We used cultured tobacco cells to prepare neutral PR-5 protein to investigate antimicrobial activities (Fig. 2C). Further determination of high-resolution crystal structure at 1.8 Å revealed that the negatively charged surface cleft of the PR-5 protein plays a role in antifungal activity.

Cultured plant cells are also useful in studying the regulation of the gene expression of PR-proteins in vitro. When the ethylene-responsive regulation of PR-5 d in cultured cells was characterized, the post-transcriptional regulation of ERF activities was also clarified in the NaCl-response. A better understanding of the intracellular sorting of PR-5 d in tobacco cells should provide basic information for the production of recombinant proteins in cell cultures.

4. Characterization of chloroplast development in photoautotrophic cells and a novel DNA-binding protease, CND41, in chloroplast gene expression and plant development

Photoautotrophically cultured cells also provide unique opportunities in studying chloroplast development, since functionally differentiated chloroplasts should divide continuously and differentiate during the growth of PA cells. This developmental condition is different from that in intact plants, in which chloroplasts generally divide as proplastids and differentiate into chloroplasts. The percentage of dumbbell-shaped chloroplasts (the intermediate chloroplast division) in photoautotrophic cells was maximal (about 23% of total chloroplasts) 3 d after inoculation, before the cells had started to grow.

In view of the advantages of cultured green cells, highly purified chloroplast nucleoids were isolated and characterized in order to investigate the regulation of chloroplast development. The chloroplast nucleoids showed run-on transcriptional activity in vitro, and a protein of 41 kDa (CND41) was identified as a major DNA-binding protein in the nucleoids of the cultured tobacco cells. Unexpectedly, the predicted amino acid sequence indicated not only the presence of a lysine-rich N-terminal region for DNA-binding, but also the presence of an aspartic protease active site motif in CND41. Further analysis of transgenic tobacco with reduced CND41 suggested that the possible function of CND41 as a negative regulator of chloroplast gene expression.

We also found the effect of CND41 on plant growth. The reduction of CND41 in an antisense transformant not only accelerated plastid development in shoot apex cells and early young leaves, but also brought about a dwarf phenotype and altered leaf morphology. Plant height and leaf shape were restored almost to the same as those in the wild type by the application of gibberellins (GAs), which indicates that a reduction in GA content was a prime cause of the dwarf phenotype in the CND41 antisense transformants. The endogenous levels of active gibberellin (GA$_1$) in transformants were lower than those of wild-type plants. This was the first evidence of a link between plastid function and plant development through plant hormone biosynthesis.

Protease activity was further confirmed with CND41 purified from cultured tobacco cells. Characterization of transgenic tobacco by altered expression of CND41 clarified its physiological importance in vivo, in that senescence was delayed in transgenic tobacco with reduced expression of CND41. Additionally, nitrogen-depletion experiments on low-CND41 transgenic tobacco clearly indicated that CND41 plays an important role in the regulation of senescence and the re-allocation of nitrogen through degradation of denatured ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco). CND41 is the first specific protease found to play a role in Rubisco degradation during senescence, although autophagy has also been found to regulate senescence. Strict regulation of the protease activity of CND41 in tobacco that overexpresses CND41 under healthy growth conditions has yielded insight into the regulation of senescence when CND41 is developmentally processed for protease activity.

5. Further characterization of photosynthetic functions: oxygen evolving complex, especially a 23-kD protein (PsbP) in extrinsic protein complex, and the cyclic electron transfer system

Isolation of salt-tolerant PA tobacco cells provides another opportunity for studying the importance of photosynthesis under stress conditions. Salt-tolerant cells clearly showed higher photosynthetic activities on a chlorophyll basis, especially the activities of photosystem II (PSII), against a higher concentration of NaCl than control. Further experiments showed a stronger association of a 23-kD protein (PsbP) in oxygen evolving complex with PSII membranes.

To determine the role of PsbP in PSII functioning, the PsbP activity in PSII was characterized biochemically by reconstitution experiments. Furthermore, the 3-dimensional structure of PsbP was determined at 1.6 Å. Based on a biochemical analysis of PsbP functioning, transgenic tobacco plants with reduced accumulation of PsbP isoforms with isogene-specific trigger RNA for each PsbP gene were established, and their photosynthetic properties were determined. With complementary transformation of the extra PsbP genes without the target sequence for RNAi into the silenced plants, a linear correlation was noted between PSII activity and the total amount of PsbP. Further characterization of the PsbP-deficient plants indicated that PSII without PsbP was hypersensitive to light, and...
was rapidly inactivated when repair of damaged PSII was inhibited by chloroplast transcription inhibitor chloramphenicol. All of these results indicate that PsbP plays an indispensable role in the regulation of photosynthetic electron transfer in intact plants.

Characterization of the PsbP gene family also revealed considerable functional diversification in this protein family as an essential factor in PSI, PSII repair, and cyclic electron transfer around photosystem I.42-43 Especially, cyclic electron transfer around photosystem I has attracted considerable attention due to its essential role in normal photosynthesis,44 environmental responses under stress conditions,45 and C4 photosynthesis.46 However, the function of CET in PA cells remains unclear. Readers interested in the details of CET should consult further reviews.47,48

As mentioned above, cultured PA cells yield considerable information about the cellular development of chloroplasts, photosynthesis, and other plant functions in vitro. Whereas some stress responses of plant cells are expressed under normal culture conditions without special application of exogenous stress, the photoautotrophic nature of these cells expands experimental opportunities in photosynthesis research. Recently, a photoautotrophic Arabidopsis thaliana cell culture that does not require additional enrichment with carbon dioxide was established (Takeda and Sato, unpublished data). This cell line should provide further opportunities to study photosynthesis under more natural carbon dioxide conditions.

II. Molecular Characterization of the Benzylisoquinoline Alkaloid Biosynthetic Pathway and Its Biotechnological Applications Using Alkaloid-Producing Cultured Plant Cells

1. High berberine-producing cultured Coptis japonica cells and molecular characterization of berberine biosynthesis

The establishment of cell cultures that produce high levels of secondary metabolites can offer clear advantages over slow-growing medicinal plants. Selected cultured cells show high metabolite productivity with high activities of biosynthetic enzymes, little tissue differentiation, no seasonal variation, and little variation among samples. Thus cells that produce high levels of secondary metabolites considerably enhance the study of secondary metabolism.49,50

Coptis japonica, a member of the Ranunculaceae, is a commonly used natural medicinal plant in Japan, China, and Korea. It is listed in the Japanese Pharmacopoeia.51 Since naturally grown rhizomes require 5 to 10 years before harvest, there have been many studies on the development of alternative methods for the production of Coptis rhizomes. While the Coptis rhizome is used as a crude drug for the extraction of a mixture of benzylisoquinoline alkaloids, the main active compound, berberine, is also useful as an antimicrobial and anti-inflammatory agent, and it has attracted considerable attention due to its lipid-reduction activity in animal cells.52

Coptis japonica cells with high berberine-productivity have been established by cellular selection, as in the case of PA cells,53 but the characteristics of crude drug materials that contain multiple constituents with opposite, moderating, or enhancing effects make it difficult to identify the main constituents and mechanisms, which means that it is difficult to identify associations between cultured medicinal plant cells and crude drugs. Hence, cultured C. japonica cells are not yet suitable as alternatives to Coptis rhizome. On the other hand, the strong berberine-producing activity of cultured cells by itself is not sufficient for the preparation of pure berberine as a medicine due to the cost of purifying berberine from a mixture of related alkaloids. Hence cultured cells currently cannot be used as a simple source of medicines, except for invaluable medicines such as paclitaxel.54 Nevertheless, metabolite-producing cell cultures are useful for the study of biosynthetic enzymes for these metabolites (Fig. 3).49,50

Since the biosynthetic enzymes in the berberine biosynthetic pathway are highly expressed in C. japonica cells that produce large amounts of berberine, several biosynthetic enzymes in the benzylisoquinoline alkaloid pathway, including scoulerine 9-O-methyltransferase (SMT),55,56 norkochinidine 6-O-methyltransferase (6OMT)/3'-hydroxy-N-methylcoclaurine 4'-O-methyltransferase (4'OMT),57 and coclaurine N-methyltransferase (CNMT),58,59 have been purified, and their enzymological properties have been determined. The isolation of their cDNAs based on the amino acid sequence of purified enzymes has provided molecular tools for investigating enzymes that are structurally similar but difficult to purify by ordinary column chromatography (e.g., 6OMT and 4'OMT).60

Selected C. japonica cells with high berberine-productivity proved useful in the isolation of the cDNAs of other membrane-bound enzymes, such as cytochrome P450 proteins (P450s). While P450s are highly diversified super-family in plants,61 mainly two P450 species have been expressed selectively in C. japonica cells that produce large amounts of berberine: (S)-N-methylcoclaurine-3'-hydroxylase (CYP80B2) and canadine synthase (CYP719A1).62 CYP719A1 catalyzes the conversion of (S)-tetrahydrocolumbamine to (S)-tetrahydroberberine (canadine) via methylenedioxy-bridge formation. It was the first cDNA to be isolated for this common reaction in plant metabolites.

Another CYP719A1 subfamily isolated from Eschscholzia californica cells also showed methylenedioxy-bridge formation activity, with slightly different substrate- and regio-specificities.63,64 but methylenedioxy-bridge formation is not restricted to the CYP719 family, since an enzyme that promotes methylenedioxy- ring formation in lignan biosynthesis, sesamine synthase, was isolated and identified as CYP81Q1.65 Currently available information indicates that all members of the CYP719 family participate in benzylisoquinoline biosynthesis and are good molecular markers of chemotaxonomy, although they show different reaction activities.63,64 In fact, CYP719B1, isolated from opium poppy, does not catalyze methylenedioxy-ring formation, but rather catalyzes intramolecular C-C phenol coupling of (R)-reticuline to salutaridine in morphine biosynthesis.66

An EST library of C. japonica cells with high berberine-productivity limited to 5,000 entries was also useful in isolation of the remaining unidentified biosynthetic
Fig. 3. Benzylisoquinoline Alkaloid Biosynthetic Pathway Characterized in Cultured *Coptis japonica* (Cj) and California Poppy (*Eschscholzia californica*: Es) Cells.

NCS, (S)-norcoclaurine synthase; 6OMT, norcoclaurine 6-O-methyltransferase; CNMT, (S)-coclaurine N-methyltransferase; CYP80B1, (S)-N-methyllcoclaurine 3'-hydroxylase; 4'OMT, 3'-hydroxy-N-methyllcoclaurine-4'-O-methyltransferase; BBE, berberine bridge enzyme; SMT, (S)-scoulerine 9-O-methyltransferase; CYP179A1, canadine synthase ((S)-tetrahydroberberine synthase); THBO, tetrahydroberberine oxidase; CoOMT, columbamine-O-methyltransferase; CYP80G2, corytuberine synthase; CYP179A5, cheilanthifoline synthase; CYP179A2/A3, stylopine synthase; 7OMT, (S)-reticuline 7-O-methyltransferase. The cDNAs of the enzymes shown in red were first isolated in our laboratory, and those in blue were isolated as described in the literature.

Fig. 4. Outline of Metabolic Engineering in California Poppy Cells.

A, Overexpression of the rate-limiting enzyme, norcoclaurine 6-O-methyltransferase (6OMT), using *C. japonica* cDNA, in California poppy cells increased the accumulation of the main alkaloid of the cells, sanguinarine, as shown by the upward red arrow. B, Shutdown of berberine bridge enzyme (BBE) expression with RNAi vector clearly downregulated the accumulation of sanguinarine and the intermediate scoulerine, as shown by the downward blue arrow. On the other hand, RNAi of BBE induced marked accumulation of reticuline, a substrate of BBE, with an increase in the by-product, 7-O-methyl-reticuline, as shown by the upward blue arrows. C, Introduction of a branch pathway with scoulerine 9-O-methyltransferase (SMT) also induced a marked shift in the metabolic flow, as shown by the green arrows. SMT overexpression greatly decreased the accumulation of sanguinarine-type alkaloids with two methylenedioxy ring structures, but induced the production of protoberberine-type alkaloids, such as berberine and columbamine, and of allocryptopine- and chelerythrine-type alkaloids with one methylenedioxy ring and two methoxy groups.
enzymes. Thus, norcoclaurine synthase (NCS),\(^{67}\) columbamine O-methyltransferase (CoOMT),\(^{68}\) tetrahydroberberine oxidase (THBO),\(^{69}\) corysterone synthase (CYP80G2),\(^{70}\) and two transcription factors (WRKY and bHLH) were isolated and characterized.\(^ {71,72}\)

Transient RNAi with protoplasts isolated from C. japonica cells with high berberine-productivity was highly effective in the screening genes that are difficult to isolate, and especially those for transcription factors involved in berberine biosynthesis.\(^ {71,72}\) When the effects of the double-stranded RNA of about 40 candidate transcription factor genes were examined, the RNAi of two genes (CjWRKY1 and CjbHLH1) showed marked reductions in the transcripts of almost all of the genes involved in berberine biosynthesis, and there was little effect on the transcript levels of glyceraldehyde-3-phosphate dehydrogenase and chorismate mutase in effect on the transcript levels of glyceraldehyde-3-phosphate dehydrogenase and chorismate mutase in primary metabolism. On the other hand, ectopic expression of CjWRKY1 and of CjbHLH1 cDNA in C. japonica protoplasts was clearly associated with induction of the expression of all of the genes of berberine biosynthetic enzymes, whereas the levels of genes in primary metabolism were not affected. While the transcription activities of CjWRKY1 and CjbHLH1 must be regulated post-transcriptionally, a simple method for the transformation of protoplasts isolated from cultured C. japonica cells would be advantageous for future characterization of the modification of transcription factors and interacting proteins.

2. Metabolic engineering in cultured cells

Transgenic approaches are powerful tools for dissecting and modifying biosynthetic pathways for biotechnological applications (Fig. 4).\(^ {73}\) With the isolation and accumulation of molecular tools (biosynthetic enzyme and transcription factor genes) for metabolic engineering, our dream to produce useful metabolites in cell culture systems becomes more realistic. The earliest attempt to deregulate the rate-limiting step in the production of a useful metabolite was accomplished with overexpression of bottle-neck enzyme hyoscyamine 6-beta hydroxylase in Atropa belladonna plants to produce scopalamine from hyoscyamine.\(^ {74}\)

Whereas the first attempt by Yun et al. was successful,\(^ {74}\) overexpression of biosynthetic enzyme genes has often failed to overcome rate-limitation due to a paucity of information regarding biosynthetic activities in the pathway.\(^ {75}\) Hence we characterized the biosynthetic enzyme activities in the berberine pathway\(^ {76,77}\) and then examined effects of the overexpression of two O-methyltransferases (6OMT and 4′OMT) at the early steps of benzylisoquinoline alkaloid biosynthesis. Heterologous host cells of California poppy (Eschscholzia californica) were used to avoid the gene-silencing effect of endogenous genes. We also used cultured cells, since they do not exhibit organ differentiation for metabolite compartmentation and are easy to prepare for analysis.

Overexpression of C. japonica 6OMT in California poppy cells increased the average alkaloid content to 7.5 times greater than that in the wild type (Fig. 4A), whereas overexpression of C. japonica 4′OMT had only a marginal effect.\(^ {78}\) Overexpression of 4′OMT in a homologous host, the C. japonica plant, however, resulted in a marked increase in the amount of alkaloids, whereas transgenic C. japonica plants that overexpressed Cj4′OMT showed slower growth than the wild type.\(^ {79}\) Many experiments have indicated that the overexpression of other biosynthetic enzymes in various host plants has various effects, and thus suggest that more intensive studies of metabolic flow in various types of target-plant cells, are essential for continued progress in metabolic engineering, and especially to improve yields, through overexpression of rate-limiting enzymes.\(^ {73}\) Thus, cell culture systems offer another advantage in analyzing metabolic flux, since they are amenable to the application of stable isotopes.\(^ {80}\)

The value of metabolic engineering is not limited to achieving improved yields. It can also be used to improve quality, and this might have even greater potential than improved yields in, for example, metabolic diversification by the introduction of branch-pathway enzymes (e.g., SMT expression in California poppy) and/or the knockdown of enzyme expression by RNA interference (RNAi) (Fig. 4B, C).\(^ {81-84}\) The latter approach in particular can be very useful for inducing an accumulation of key intermediates by reducing main or side reaction, and then redirecting the flow to produce the desired metabolites, with additional expression of a branch- or key-pathway enzyme. In fact, the power of RNAi in suppressing gene expression and the accumulation of key intermediates, e.g., reticuline, has been demonstrated with RNAi of the berberine-bridge enzyme gene (BBE) (Fig. 4B),\(^ {84}\) while the traditional antisense method was often insufficient to induce an accumulation of reticuline.\(^ {85}\) One g of fresh weight of BBE RNAi cells secreted significant amounts of reticuline into the medium (a maximum of 6 mg/20 mL, culture medium) at more than 90% purity, while these cells contained an average of 0.06 mg, reticuline per g fresh weight. Secretion of metabolites from cultured cells with a modified metabolism into the medium has often been observed, and this might prove a useful feature in the engineering of secondary metabolism.\(^ {82,84}\)

The most interesting modification of quality was the introduction of a new branch pathway (Fig. 4C). Whereas the introduction of a pathway to produce completely novel metabolites in the cells of a host plant might prove toxic, minor modifications of the pathway, such as the introduction of protoberberine biosynthesis into the benzophenanthridine pathway, should be an acceptable modification for host cells, since benzophenanthridine alkaloids are also produced through protoberberine-type intermediates (Fig. 3). Thus the introduction of Coptis scoulerine 9-O-methyltransferase (CjSMT) into E. californica cells modified the metabolic flow, and these cells accumulated tetrahydrocolumbamine and columbamine as novel metabolites. Unexpectedly, the high adaptability of plant metabolism allowed the redirection of these modified metabolites into an endogenous benzophenanthridine pathway, producing allocryptopine, chelerythrine, and 10-methoxychelerythrine in transgenic California poppy cells that overexpressed CjSMT, while protopine, sanguinarine, and chelirubine were found in control cells (Fig. 4C).\(^ {82}\) This robust metabolism should help plant cells, to adapt to biochemical changes, and cultured plant cells are a suitable system for testing evolutionary diversification, since they
have strong potential for somaclonal variation.\textsuperscript{78,86} These results also suggest that enzymes in secondary metabolism might have broad substrate-specificity and high adaptability to metabolic modification induced by spontaneous genetic modification. Cultured cells with a modified metabolic pathway might be useful in tracing the evolution of metabolism.

3. Beyond metabolic engineering

Metabolic engineering is a powerful tool for modifying secondary metabolism in plants, but the highly mixed constituents of plant metabolites might be too complicated for the production of any specific chemical, and plants and cultured plant cells often grow too slowly for industrial applications. Thus, when more cDNAs of biosynthetic enzymes become available, the reconstruction of an entire pathway by synthetic approaches might be useful for producing plant metabolites more efficiently based on a redesigned pathway in microbes (Fig. 5).

In fact, the reconstruction of biosynthetic processes in an entire plant has been examined in microbial systems.\textsuperscript{87,88} In alkaloid biosynthesis, two groups have reconstructed a benzylisoquinoline alkaloid pathway to produce reticuline from simple precursor dopamine or norlaudanosoline in \textit{E. coli} and in budding yeast.\textsuperscript{89,90} Furthermore, fermentative production of reticuline from simple carbon sources, such as glucose and glycerol, has been achieved using a modified \textit{Escherichia coli} platform.\textsuperscript{91} These advances might lead to a new era in the biosynthesis of natural plant products, but they do not mean that cultured plant cells are obsolete, since synthetic biology is still very primitive, and more information is needed on the biological aspects of secondary metabolism. More detailed procedures, including the compartmentation of biosynthetic enzymes in plant cells and more biosynthetic enzymes for more complicated pathways, are needed, while some membrane-bound and vesicle-localized enzymes are expressed in eukaryotic microbial cells such as yeast and used for reconstruction (Fig. 5).\textsuperscript{89,90}

III. Perspectives

This review presents the advantages and disadvantages of cultured plant cells that exhibit functional differentiation. While cultured plant cells are certainly different from somatic cells in intact plants, functionally differentiated cells represent unique materials for studying plant cell functions, as discussed above. However, the functionally differentiated plant cell cultures established thus far are very limited in number. With regard to photoautotrophism, only C3 photosynthetic cells have been established in vitro, and no C4 photosynthetic cells or organ cultures have been established.\textsuperscript{51} Similarly, only limited number of secondary metabolite-producing cell cultures have been established. Thus, no morphine-producing poppy cell culture has been reported.\textsuperscript{49} As recent molecular studies have indicated, cellular differentiation is controlled by inter-cellular signaling as well as endogenous developmental control (e.g., the developmental control of stomata through stomagen and of shoot apical meristem through CLE peptides).\textsuperscript{92,93} While it is not yet possible to recreate this cellular differentiation in vitro using inter-cellular signaling networks, a cell culture system is crucial for evaluating the factors that are involved in cellular networks.

Another challenge is to regenerate whole plants from functionally differentiated cells with beneficial mutations or modifications such as high metabolite productivity or herbicide resistance. Whereas a mutation in the nuclear genome can be transformed through genetic engineering, engineering of the plastid/mitochondria genome is still difficult even given advanced transformation technology.\textsuperscript{94,95} Similarly, the regeneration of high metabolite-producing cells should provide much deeper insight into the regulation of metabolism, and should answer fundamental questions as to how the expression of genes in secondary metabolism is integrated into the entire plant, and also how the secondary metabolism in a plant functions under natural environmental conditions. Cell culture systems clearly complement transgenic approaches and should be useful in the future development of plant biotechnology.

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