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Pharmaceutically Active Natural Product Synthesis and Supply via Plant Cell Culture Technology

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Abstract: The chemical diversity of plant-derived natural products allows them to function in a multitude of ways including flavor enhancers, agricultural chemicals, and importantly, human medicinals. Supply of pharmaceutically active natural products is often a challenge due to the slow growing nature of some species, low yields found in nature, and unpredictable variability in accumulation. Several production options are available including natural harvestation, total chemical synthesis, semisynthesis from isolated precursors, and expression of plant pathways in microbial systems. However, for some medicinal natural products, such as the anticancer agent paclitaxel, where low yields in nature, chemical complexity and lack of knowledge of the complete biosynthetic pathway, preclude many of these options, plant cell culture technology is an attractive alternative for supply. Plant cell suspension cultures are amenable to scale-up, environmental optimization, and metabolic engineering. This review focuses on some of the key challenges in utilizing and commercializing plant cell culture suspension technology, with a focus on pharmaceutically active natural products. Recent research has been directed toward application of traditional strategies such as reactor design, cell immobilization, and enzyme elicitation as well as emerging strategies such as characterizing cellular heterogeneity and variability through flow cytometric techniques, metabolic engineering, and system-wide analysis.

Keywords: Plant cell culture; metabolic engineering; paclitaxel; secondary metabolite; flow cytometry

Introduction

Plants are a tremendous source of natural diversity in the multitude of compounds that they synthesize, many of which have been shown to be useful as therapeutics for treating a variety of human illnesses and maladies. In fact, it has been estimated that approximately two-thirds of the active ingredients in anticancer drugs and drugs for infectious diseases are plant-derived,[1] even though only a fraction of all naturally occurring chemicals have been investigated. Most of these active ingredients can be classified as secondary metabolites, broadly defined as any product which a plant synthesizes that is not necessary for basic life functions such as growth or replication. Secondary metabolites are a large, varied, and sometimes mysterious group of molecules; while some are most likely extraneous byproducts of metabolic pathways due to promiscuous enzyme activity, many do serve important functions in defense or protection *in planta*, and thus can be considered biologically active. It is a result of this biological activity that these molecules can be useful as applied treatments for human ailments; this has been recognized for centuries as plants have been used in traditional medicine across the world. Over the past 100 years, as specific metabolites have been chemically isolated and identified, commercial production of these biologically active phytochemicals represents an enormous challenge. Most secondary metabolites are often present in extremely

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low amounts in the plant, often less than 1% of the total carbon.[2] This paucity can make natural harvestation impractical for bulk production, especially in the case of slow growing species. The significant engineering challenge is then to find a means by which to produce the desired natural products in a way that is both sustainable and financially feasible. Production of these products in a microbial or fungal host by transferring the biosynthetic pathway is possible, but is limited due to the complexity and lack of complete knowledge regarding many of these pathways (see reviews in this issue). This review will highlight production options from plants, with a specific focus on plant cell culture technology.

Production Options for Natural Products

Chemical synthesis of natural products is possible and commercially feasible, particularly for those with relatively simple chemical structures such as aspirin (derived from the natural product salicylic acid) and ephedrine.[3] In many cases, however, the metabolite has a complex structure, which can include multiple rings and chiral centers, so that a synthetic production process becomes prohibitively costly. Many natural products used in cancer treatment, including compounds such as paclitaxel, vinblastine, and camptothecin, fall into this latter class, so an alternative method of supply is necessary.

Depending on the nature of the plant, extraction directly from harvested plant tissue may be an option. Especially if a plant can be cultivated en masse, this can be attractive on a commercial basis. The anticancer drugs vincristine and vinblastine, among other medicinally valuable metabolites such as ajmalicine and serpentine, are found in the Madagascar periwinkle *Catharanthus roseus*.[4] Even though these important alkaloids, particularly vincristine and vinblastine, naturally occur at very low levels in *C. roseus*—less than 3 g per metric ton—the fast growing nature of the periwinkle makes field cultivation most practical at the present time.[3] However, the relative inefficiency and high cost of whole plant extraction implies that an improved method of supply would be useful for these valuable anticancer agents.

When natural supply is limited due to a combination of low yields and slow growth rates, in vitro cultures provide an attractive alternative. Most plant species can be cultured in vitro in either an undifferentiated or differentiated state. As many secondary metabolites are produced by specialized cells, organ cultures such as shoots or roots can exhibit similar metabolite profile patterns compared to the native plant,[5] whereas undifferentiated cultures often accumulate secondary metabolites to a lesser extent, and sometimes not at all. The anticancer compound camptothecin, produced by the ornamental tree *Camptotheca acuminata* as well as *Nothapodytes fetida* and *Ophiorrhiza pumila* among other species, has been shown to accumulate in undifferentiated cultures in very low or even undetectable amounts,[6] compared to root cultures in which production levels were comparable to the intact plant.[7] Similarly, no artemisinin, a potent antimalarial drug, was found in cell suspension cultures of *Artemisia annua*, while trace amounts were detected in shoot cultures.[8] Root cultures can be transformed into hairy roots using the soil dwelling bacteria *Agrobacterium rhizogenes*, resulting in cultures which are genetically stable, capable of unlimited growth without additional hormones, and have an increased capacity for secondary metabolite accumulation (reviewed most recently in refs 9 and 10). The commercial potential of hairy root cultures has been limited primarily due to challenges in cultivating hairy roots in a large scale system. Hairy roots form a complex, nonhomogeneous, solid matrix, and present enormous difficulties in terms of bioreactor configuration.[9] While significant effort is currently focused on novel approaches to bioprocess and reactor design, such as a system in which roots are grown on racks and continuously sprayed with medium[11] and an acoustic mist bioreactor in which a nutrient mist is generated by an acoustic field,[12] difficulties associated with scale up have generally prevented widespread commercial application of this technology.

Undifferentiated suspension cultures, which can be more easily scaled to levels suitable for commercial production, have been studied for producing useful metabolites since the

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Table 1. Commercial Plant Cell Culture Processes for Supply of Natural Products with Pharmaceutical Applications (Adapted from Ref 14)

metabolite	species	application	manufacturer
scopolamine	*Duboisia* spp.	anticholinergic	Sumitomo Chemical Industries (Japan)
protoberberines	*Coptis japonica* *Thalictrum minus*	antibiotic, anti-inflammatory	Mitsui Petrochemical Industries (Japan)
paclitaxel	*Taxus* spp.	antitumor	Phyton Biotech (USA) Samyang Genex (Korea)
rosmarinic acid	*Coleus blumei*	anti-inflammatory	Nattermann (Germany)
ginseng	*Panag ginseng*	dietary supplement	Nitto Denko (Japan)
Echinaceae polysaccharides	*Echinacea purpurea* *Echinacea augustifolia*	anti-inflammatory, immunostimulant	Diversa (Germany)
shikonin	*Lithospermum erythrorhizon*	anti-HIV, antitumor, anti-inflammatory	Mitsui Petrochemical Industries (Japan)
geraniol	*Geramineae* spp.	antitumor	Mitsui Petrochemical Industries (Japan)

1950s with mixed success. There are currently 14 plant cell culture processes which have been commercialized for production of secondary metabolites[13] (including products used in applications other than pharmaceuticals such as food and cosmetics).

Table 1 presents an overview of secondary metabolites specifically used in pharmaceutical applications produced on a commercial scale. There are relatively few commercial processes due in part to the fact that many metabolites simply do not accumulate in undifferentiated cultures. Presumably, the biosynthetic pathways for many secondary metabolites are under strict control during developmental regulation leading to spatial separation of pathway segments in different types of tissues. In *A. annua*, artemisinin has been shown to accumulate at different levels in shoots, seeds, leaves, and flowers, with the highest levels in the flowers.[15] Undifferentiated cultures lack these specific organs or tissues, which may be associated with critical portions of the biosynthetic pathways. Additionally, compartmentalization of secondary metabolite biosynthetic pathways also occurs at the subcellular level, and cells cultured *in vitro*, which have higher growth rates than soil-grown plants, may lack fully developed compartments such as plastids and vacuoles, which most likely contribute to the lower productivity of these cultures.[16] Even in those cultures which do produce the compound of interest, yields are often low and highly variable,[17,18] and most engineering work is focused on understanding and improving limitations associated with these two factors. Despite the challenges of using plant cell suspension cultures, advantages such as ease of scale-up and simpler purification schemes due to product secretion have prompted extensive research into facilitating commercialization of this technology.

Plant Suspension Cell Culture Technology

Production of metabolites via plant cell suspension culture is renewable, environmentally friendly, and from a processing standpoint, amenable to strict control, an advantage in regards to meeting Food and Drug Administration manufacturing standards. Technology developed for other cell culture and fermentation systems (e.g., mammalian and yeast) can be readily adapted for large scale applications with plant cells, easing difficulties associated with scale-up. A notable example of the success of plant cell culture systems, due in large part to innovative research and the application of novel technologies, is paclitaxel synthesis and supply. Paclitaxel, produced by *Taxus* spp., is an important anticancer agent used as a first line treatment for several types of cancer, including breast, ovarian, and nonsmall cell lung cancer, and has also shown efficacy against AIDS-related Kaposi sacoma.[19] Production of paclitaxel via cell culture technology has been studied since the 1980s as an alternative supply source to harvest of the slow growing yew tree, since a single dose of 300 mg requires the sacrifice of a 100 year old tree.[20] A combination of process engineering and directed biosynthesis approaches ultimately led to significant improvement of yields in cell culture systems, to the extent that commercial

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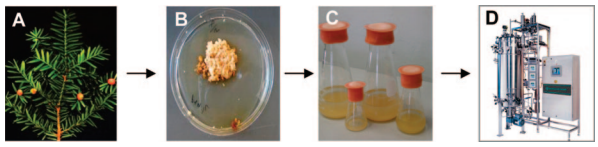

Figure 1. Initiation of a plant suspension cell culture from explant material. Plant tissue (A) plated on solid growth induction medium will yield a callus culture (B), which can then be transferred to a liquid medium to obtain a suspension culture (C) and ultimately scaled-up for commercial production (D).

success has been achieved by Phyton Biotech, in supplying Bristol-Myers Squibb paclitaxel for its Taxol formulation.[21]

Suspension cell cultures are initiated from an explant that has been isolated from plant material (e.g., embryo, needle, bark, stem). This explant can be plated on a solid growth medium (Figure 1), which must be tailored specifically for different species. Growth medium typically consists of a carbon source, minerals, phytohormones, and antioxidants. Under suitable conditions, the explant will grow into a proliferating mass of undifferentiated cells known as a callus culture. The callus can subsequently be transferred from solid to liquid medium, resulting in a suspension cell culture that is incubated under agitation and controlled temperature. The undifferentiated cells are considered to be totipotent—that is they have the ability to differentiate into any somatic cell type, and under suitable conditions, these cultures can be used to regenerate fertile plants in most species. However, this undifferentiated state is not stable, and evidence suggests that all cultures lose the ability to regenerate into plants over time.[22] To this point, little is known about how the developmental state of cells change over time in culture, which is of particular interest concerning the long-term maintenance of suitable cell lines used for metabolite production (see below).

The primary challenges impeding regular commercial application of plant cell culture technology are low and variable yields of metabolite accumulation. As mentioned above, some metabolites do not accumulate in appreciable quantities in undifferentiated cells. In these cases, manipulation of genes within the biosynthetic pathway is needed to utilize plant cell cultures for bulk production, which is often unrealistic due to a lack of complete knowledge regarding secondary metabolic pathways and their regulation in most plant systems (see below). In the case that metabolite accumulation occurs in low yields, traditional strategies based on similar approaches in other types of cell culture and fermentation systems have been successful in improving metabolite yields to suitable levels for commercial production, as described below. Controlling variability in product accumulation has often been neglected in favor of improving yield, but more recently, the recognition of variability as a key limiting factor,[23] especially in the common case in which metabolite accumulation decreases as a cell line ages[24–26] has led to a renewed focus on understanding its basis as a fundamental goal in current plant cell culture research. This review highlights current research aimed at both traditional and nontraditional approaches to improving both yield and stability.

Traditional Strategies To Improve Cell Culture Yields

To date, much of the work on plant cell cultures that has translated into commercial success involves optimization strategies similar to those developed for other cell culture and fermentation processes. This type of process engineering approach includes manipulation of culture operating parameters such as media composition, cell line selection, and gas phase composition (reviewed in refs 27 and 28). These strategies are a necessary starting point in many cases, especially when initiating a new cell line. The following section presents a brief overview and recent applications of some approaches that have traditionally been of interest to plant cell culture process development. While many of these ideas may be considered outdated in favor of newer metabolic engineering perspectives, their successful application is essential for the eventual success of plant cell culture technology and, therefore, remain active areas of research.

Perhaps the most notable strategy for improving metabolite yields is elicitation. An elicitor can be defined as any compound that induces an upregulation of genes. Some elicitors target secondary metabolic genes, which are often associated with defense responses to perceived environmental changes. Elicitors include natural hormones, nutrients, and many fungi-derived compounds. In particular, jasmonic acid and its methyl ester methyl jasmonate (MJ), are naturally occurring hormones involved in the regulation of defense

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genes as part of a signal transduction system.[29] Applied exogenously, they have been shown to induce secondary metabolic activity and promote accumulation of desired metabolites in numerous plant systems, including *Taxus* spp.[30,31] and *C. roseus*.[32,33] Different elicitors may act on different segments of the biosynthetic pathway. For instance, MJ elicitation compared to salicylic acid elicitation in *Taxus* spp. cultures resulted in different relative increases of metabolic intermediates,[34,35] suggesting that each elicitor preferentially directs flux toward, and possibly away from, different intermediate taxanes. While many of the specific targets of elicitors have yet to be conclusively identified, elicitation can be an extremely useful tool in conjunction with gene expression profiling for identifying rate-influencing steps in secondary biosynthetic pathways (see below).

Product removal in situ has received considerable interest over the years, especially when using transgenic plant systems for the expression of foreign proteins which may be degraded post synthesis.[36] Metabolite accumulation in cell cultures may be limited by feedback inhibition and product degradation, so "two-phase" systems present obvious advantages, including simpler downstream recovery. Many secondary metabolites may also be toxic to cultures at artificially high levels induced by elicitation, making product removal necessary for continued growth and biomass accumulation. More recently, the use of extraction resins and adsorbents has been shown to increase productivity in several systems, including anthraquinones from *Morinda elliptica*[37] and ajmalicine from *C. roseus*.[38] A combination of an external extraction column with a high-rate perfusion bioreactor has been developed[39] in a scalable design, indicating the practical applicability of this approach.

Immobilization of plant cell cultures has long been considered for increasing metabolite accumulation, as the potential of higher cell densities, continuous removal of products/inhibitors, and protection for shear-sensitive plant cells provide a number of advantages.[40] Immobilization can be simply achieved using a gel matrix such as alginate; however this becomes costly at a larger scale, especially when the product of interest is not secreted and must be released using sonication or treatments with an organic solvent.[5] Recently, immobilization of *T. baccata* cells in calcium-alginate beads was shown to produce one of the highest reported levels of paclitaxel accumulation among academic laboratories (43 mg/L).[41] Immobilization also has the potential to simplify product extraction and purification, as immobilized cultures of *Linum usitatissimum* excrete the pharmaceutically active metabolite dehydrodiconiferyl alcohol-4-β-D-glucoside (DCG) to a greater extent than suspension cultures.[42]

Plant cell suspension cultures in large-scale bioreactors are subject to the hydrodynamic forces resulting from mechanical agitation, and many reactor designs have been suggested over the years for minimization of these detrimental effects, often using airlift or bubble column designs to replace mechanical impellers.[2] Plant cells are much larger than mammalian cells or microbes which make them extremely susceptible to shear forces in the surrounding fluid. Different types of plant cells exhibit different responses related to shear forces, and detailed studies have been performed on individual species evaluating a variety of effects relative to shear forces, including reduction in viability, release of intracellular components, changes in metabolism, and changes in morphology.[43] Generally, exces-

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sive shear forces can lead to cell lysis and reduced viability. More recently, efforts have been focused on understanding the processes and underlying mechanisms involved in the cellular responses to shear. Traditionally, research focused on understanding cellular response in order to optimize bioreactor designs. Current research is being directed toward metabolic modification by either genetic transformation or optimizing specific environmental conditions, to allow cells to be less vulnerable to the negative effects of shear forces. Plant cells may actually adapt to high shear environments over time. For example, suspension cultures of *T. cuspidata* show significant differences in response to shear stress at different culture ages, as measured by reactive oxidative species (ROS) concentration, extracellular pH, and membrane fluidity, all of which have been previously shown to be early defense responses to mechanical stress.[44] Nitric oxide (NO), in combination with ROS, triggers biological responses including host cell death. NO generation and the suppression of glutathione S-transferase, a critical enzyme responsible for eliminating ROS, have been strongly correlated to shear stress in *T. cuspidata* cell suspensions in a Couette-type reactor.[45] Furthermore, the mechanism of plant cell mechanoreception appears to be analogous to integrins, which serve a similar function in animal cells through recognition of a family of extracellular glycoproteins via an Arg-Gly-Asp (RGD) motif. Based on studies which utilized a synthetic RGD peptide to effectively disrupt communication between the extracellular matrix and cell interior, as evidenced by monitoring physiological responses to shear stress, such as ROS, the existence of similar RGD binding proteins in plants has been proposed.[46] No homologue to integrin has been identified in the model plant *Arabidopsis thaliana* as of yet, but the eventual identification of the molecular structure and function of these unknown proteins in plant cells may provide an opportunity to regulate cellular physiological response to mechanical stress.

Efficient bioprocessing technologies will aid in the commercialization of plant cell culture processes. The high costs of industrial biomanufacturing facilities are due in large part to the expensive equipment used in culture processes, such as stainless steel bioreactors and support equipment, as well as the demands associated with aseptic processing, including clean utility generation and equipment for cleaning and sterilization. Disposable technology is increasingly being used in the scale up stages of industrial cell culture processes,[47] but relatively few studies have examined the suitability of this equipment for plant cell cultures. Disposable reactors are typically presterilized plastic reactors that eliminate the need for separate cleaning and sterilization cycles, and can reach working volumes of up to several hundred liters. Two types of these reactors: a wave undertow reactor consisting of a flexible plastic container on a horizontal platform that is intermittently raised to induce wave formation, and a slug bubble column consisting of a flexible, vertical cylinder in which large bubbles are periodically generated that rise to the top of the column, were recently evaluated using soya and tobacco cell cultures.[48] Apparent growth rates for both culture systems in reactors up to 100 L were comparable to those obtained in Erlenmeyer flasks and a stirred-tank bioreactor, and oxygen transfer rates were also relatively high, pointing toward the suitability of these designs for high density cell cultures. Shear forces are drastically reduced in these disposable designs, especially in the wave bioreactor in which sufficient oxygen transfer is achieved without a mechanical impeller or sparge gas aeration.

Toward Understanding and Controlling Variability in Product Accumulation

Much improvement has been made in increasing yields of secondary metabolites which accumulate at low levels. However, relatively little progress has been made in understanding and controlling the unstable secondary metabolite production patterns. The maintenance of consistently high production levels has proven to be difficult, and gradual loss of secondary metabolite productivity over time has long been known as an obstacle in the development of commercial plant cell culture production systems.[24,26] Unlike mammalian cells, which are routinely stored in liquid nitrogen, relatively few storage methods have been developed for plant cells, and those methods that do exist are typically characterized by low viability and long lag periods before recovering a rapidly growing suspension culture.[49] While cryopreservation methods can be optimized by using cryoprotectants, efficient freezing programs including cold acclimation and vitrification, and adding components such as calcium (see, for example, refs 50 and 51), most cell lines are maintained via periodic subculture. As a cell line effectively ages, the loss of desirable characteristics and instability of secondary

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metabolism is likely caused by a combination of factors, including: mutations that are likely to predominate in cell populations that have been repeatedly propagated in an undifferentiated state, varying ploidy levels which have been shown to affect secondary metabolism in several systems, occurrence of distinct subpopulations within a culture related to prolonged exposure to microenvironments within the culture system, and inherent variability associated with the repeated subculture process (see below for more detail). In particular, the implications of subpopulations related to culture behavior have received considerable interest in recent years, and is the focus of much work in our laboratory.

Plant cell suspension cultures consist of undifferentiated cells derived from explant tissue. While these cultures can be indefinitely maintained in a generally undifferentiated state with the addition of phytohormones to the media, such as auxins and cytokinins, they are not maintained in a state of genetic or epigenetic stability. Older cultures of *Nicotania plumbaginifolia* exhibited faster growth rates than newer cultures, which is attributed to the high proportion of cells with mutations that elevate cyclin dependent kinase (CDK) activity.[22] This type of mutation accelerates proliferation and obstructs processes related to organ development and differentiation, to the point that older cultures were incapable of regenerating into plants. As many metabolic pathways are compartmentalized, particularly to the plastids,[52] the lack of fully developed organelles could have profound effects on the ability of cells to operate and regulate these pathways.

Ploidy levels are commonly known to be variable in plants, and suspension cultures exhibiting varying degrees of polyploidy have been linked with the ability of a cell to differentiate[53] and regenerate into shoots.[54] Increasing ploidy levels have been correlated with gene silencing in several plant systems.[55] Similar epigenetic silencing of regulatory genes may contribute to variable accumulation of secondary metabolites in *Taxus* spp.[56] and may help to explain the relationship between ploidy variation and metabolite accumulation. Hypericin content in *Hypericum perforatum* decreased with increasing ploidy, particularly between diploid and polyploid populations,[57] whereas tetraploid cultures of *A. annua* hairy roots had a higher specific artemisinin concentration, which was partially offset by a slower growth rate.[25] Studies aimed at investigating the long-term genomic stability have revealed that a majority of *Taxus* cell lines were putatively aneuploid over a two year period.[58] As much of the ploidy variation is cyclic, it may correlate with the short-term irregular cycling patterns of metabolite accumulation often observed[59] before accumulation gradually disappears.[26] Differences in ploidy level were manifest in segments of the total cell population, as it was found that *Taxus* subpopulations with different ploidy levels coexisted in culture for extended periods of time.[58] Studies relating ploidy levels to metabolite accumulation only indirectly correlate the effect of aneuploidy with secondary metabolic activity, since metabolite accumulation is taken as a culture average, rather than relative to the individual subpopulations. Ploidy variations can be induced using mitotic inhibitors[60] and by altering plant growth regulators,[61] providing another method to alter cell culture dynamics. A more fundamental understanding of the relationship between ploidy and secondary metabolism may prove to be beneficial to design a strategy aimed at controlling metabolism through manipulation of ploidy levels.

The periodic subculture process by which all in vitro lines are maintained also contributes to some degree of variability. After controlling for different types of media, inoculum size, and flask variation in *Taxus* cultures, variability in both growth and secondary metabolism was still present, leading to the conclusion that variability is inherently induced by the subculturing process.[62]

A more mechanistic understanding of culture instability is necessary before variability in secondary metabolism can be adequately addressed. Metabolic instability is clearly related in some way to the genetic and epigenetic factors mentioned above, and analysis of specific secondary path-

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ways, such as the paclitaxel pathway (see below), at the level of gene expression is needed to better understand the evolution of metabolic regulation as a culture ages. Gene expression profiles at different time points, especially at time points which correspond to high or low metabolite production levels in cells of the same lineage, will provide better clues as to what specific genes may be affected by the aforementioned factors. These genes could then be considered as objectives for targeted metabolic engineering, to stabilize productivity over time. Our laboratory has recently profiled gene expression in paclitaxel accumulating *Taxus* cell lines;[63] a similar study is underway using a cell line with altered paclitaxel accumulation profiles.

Cellular Heterogeneity as a Basis of Variability

Most research focused on understanding culture variability relies on measures of culture averaged parameters, such as cell density and metabolite concentrations. These types of measurements are essentially averages of properties over millions of cells, and neglect variation at the single cell level; while all cell cultures display some extent of heterogeneity as a result of normal statistical distributions, distinct subpopulations within cultures have been identified that have profound implications on culture behavior. For instance, differences in bulk anthocyanin production in *C. roseus* cultures were primarily dependent on the percentage of producer cells rather than the production level of each cell, determined by visual identification of the colored pigment,[64] and similar findings were reported regarding paclitaxel accumulation in *Taxus* cultures using flow cytometry.[18] To predict the effect of different elictors on taxane accumulation, a subpopulation induction model was developed based on the assumption that only certain subpopulations would be stimulated by two different elicitors (methyl jasmonate and ethylene); combined with enzyme kinetics-derived relationships to describe the production rate of paclitaxel and other intermediate taxanes, the model accurately predicted trends of metabolite accumulation under specified elicitation conditions.[65] As subpopulation dynamics have been shown to exert considerable influence on culture behavior as a whole, understanding the basis of these subpopulations is key.

Due to the nature of plant cell division in which daughter cells often remain connected through a shared cell wall, aggregates of anywhere from two to several hundred cells can be found in suspension culture (Figure 2). These aggregates can reach sizes up to several mm, and result in

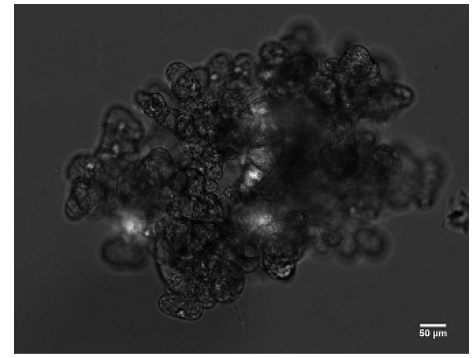

Figure 2. Aggregate of *Taxus cuspidata* P991 cells in suspension culture.

different microenvironments with respect to nutrient and oxygen availability between inner and outer regions. Cellular metabolism is altered in the presence of these differential local environments, with implications for the formation of subpopulations regarding secondary metabolite accumulation and cell proliferation. There have been conflicting reports as to the effect of aggregate size on metabolite accumulation. Studies in *Tagetes petula*[66] and *Fragaria ananassa*[67] indicate that production of secondary metabolites increases with increasing aggregate size. Jaceosidin accumulation increases as aggregate size increases in suspension cultures of *Saussurea medusa* up to 4 mm, after which jaceosidin production decreases.[68] Oxygen transport models indicate that aggregates larger than several mm face oxygen depletion at their center, which has been proposed to stimulate secondary metabolic activity directly.[66,69] Conversely, aggregation has been shown to inhibit ursolic acid accumulation in *Salvia officinalis* compared to single cells in suspension culture,[70] and aggregates were shown to synthesize about the same amount of ajmalicine in *C. roseus* suspension cultures.[69] The lack of clarity about the effects of aggregates on the culture as a whole underscores the fact that very little is known about the actual nature of the microenvironments or their effects on individual cells within aggregates.

Techniques for analyzing properties of single cells can provide insight into the nature of these aggregates by identifying subpopulations of cells from different regions with a common characteristic and can also provide informa-

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tion regarding subpopulations that may develop as a result of other factors, such as cell signaling. Flow cytometric population analysis methods have been employed by many groups studying plant cell suspension cultures, and have been used to identify subpopulations such as a group of noncycling cells in *Taxus* cultures[71] and *Solanum avicular*.[72] Noncycling cells may be specialized for secondary metabolism,[71,73] and a clearer understanding of this relationship will provide targets for strategies aimed at increasing secondary metabolite yields in culture.

Our laboratory has developed techniques for analyzing properties of individual *Taxus* spp. cells, and is continuing work to establish relationships between key cellular parameters in order to describe culture behavior in context of a mathematical framework. As plant cells grow in aggregates, methods for isolating single particles are essential for subsequent flow cytometric analysis. Protoplasts and nuclei have been prepared from many plant species for this end, including *Taxus* spp.[74,75] However, a significant amount (30–60%) of paclitaxel accumulates in the cell wall;[74] as the cell wall is removed during protoplast preparation, information regarding paclitaxel accumulation is lost. To overcome this limitation, we developed a method using the cell wall digesting enzymes cellulase and pectolyase to isolate single cells with an intact cell wall.[76] The procedure provides a high single cell yield with minimal changes in cell physiology as determined through measuring both peroxidase, a cell wall localized protein, and paclitaxel distributions before and after single cell preparation.[76] These single cells were then analyzed for paclitaxel content[18] and protein accumulation[77] using flow cytometry. A nonpaclitaxel accumulating subpopulation was identified in methyl jasmonate-elicited cultures consisting of almost 20% of the total population. Ongoing work is aimed at correlating both paclitaxel and nonpaclitaxel accumulating subpopulations with other factors, such as cell cycle participation, size, and cellular protein content.

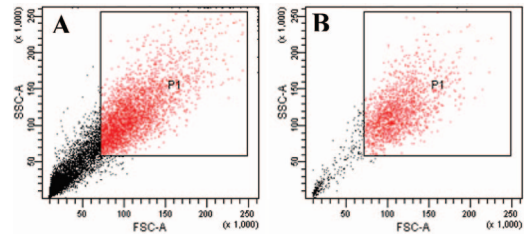

Figure 3. Unsorted (A) and sorted (B) *T. cuspidata* P991 cells. Gated region P1 was used as sorting criteria. 84% of particles fell within the P1 region of the sorted sample, compared to 36% in the unsorted sample, demonstrating that a distinct population was isolated.

These subpopulations can be isolated and further cultivated to propagate superior cell lines. Flow cytometers can be equipped with a sorting functionality, allowing for the study and reculturing of distinct plant cell populations. In particular, much effort has focused on the selection and purification of protoplasts for further experimentation in culture.[78] Under appropriate sorting and reculture conditions, protoplasts remain metabolically active, and can subsequently regenerate cell walls and begin cell division. This has been achieved with heterokaryons produced by induced protoplast fusion, as well as following transformation.[79] Similarly, our group has investigated optimal reculture conditions for isolated *Taxus* cells, and has achieved a growth rate similar to aggregated suspension cultures after using a combination of high seeding density and conditioned medium.[80]

Plant cells present several obstacles to successful sorting due to their large size. Most flow cytometers are designed to analyze and sort microbial and mammalian cells (1 – 10 μm). Because plant cells typically range from 20 to 60+ μm in size, specialized equipment and methods must be utilized. Our group has recently investigated the feasibility of sorting *Taxus* cells, initially based on size. Single cells of *T. cuspidata* were prepared following standard protocols[76] and analyzed on a Becton Dickinson (San Jose, CA) FACSVantage flow cytometer, custom equipped for sorting plant cells with a MacroSort option and 200 μm nozzle. A region defined by high values of forward scatter (FSC) and side scatter (SSC) was used as sorting criteria, and cells falling within this region were collected and further analyzed using a Becton Dickinson LSRII flow cytometer. A comparison of the sorted and unsorted samples shows that a distinct population of large cells was isolated (Figure 3). The

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primary obstacle for sorting plant cells is the instability of the flow stream when using a larger nozzle size. We have demonstrated the feasibility of sorting *Taxus* cells, which to our knowledge is the first report of sorting intact plant cells as opposed to plant derived particles such as protoplasts or nuclei. Ongoing experiments are aimed at sorting based on more relevant criteria, such as paclitaxel accumulation. Specific populations with an increased ability to produce paclitaxel can be isolated and further propagated, and eventually be used to establish stable, high-accumulating *Taxus* cell lines for use in bioprocesses. Differential gene expression analysis of high and low producing subpopulations also has the potential to lend considerable insight into the metabolic basis of different subpopulations. A fluorescence activated sorting approach has recently been used to isolate different cell types from *Arabidopsis* roots, and the resulting map of gene expression, which correlates groups of genes to specific cell fates, facilitates research on uncovering the regulatory mechanisms in organ development.[81] Similarly, expression of ROP GTPases was compared between embryogenic and pollen-like cells that were isolated using flow cytometric sorting techniques.[82]

Metabolic Engineering and Directed Biosynthesis

The engineering of biosynthetic pathways within a plant cell to enhance accumulation of a constitutively produced metabolite is an appealing strategy in which exciting progress has been made in the past decade. Secondary metabolic biosynthetic pathways are extremely complex, and still remain at least partially undefined in most cases. Few plant genomes have been fully sequenced, and those that have been are model systems in which secondary pathways are not of as acute interest regarding natural product accumulation, and as a result, a system wide analysis is sorely lacking in most medicinal plant species. Furthermore, many aspects of global metabolism remain unknown in addition to the biosynthetic pathways, including product transport and degradation, and regulatory elements such as transcription factors for pathway genes or other signaling mechanisms. Difficulties applying traditional genetic transformation methods to plant cells have impeded progress in many systems. Nonetheless, significant advancements in overcoming some of these key challenges have been made in recent years, and are highlighted in the following sections.

Metabolic Engineering Tools

A metabolic engineering approach involves the manipulation of targets within a cell. Techniques are therefore needed both for the identification of these targets (i.e., genes, proteins, metabolites) as well as for their exploitation. As many secondary pathways are still partially undefined, elucidating pathway genes and their control elements is an active research area. The subsequent identification of rate-influencing steps within a biosynthetic pathway can then be useful in providing targets for a rational engineering strategy. A variety of tools have been employed to both identify unknown genes and characterize secondary metabolite pathway regulation, including precursor feeding, gene overexpression, application of metabolic inhibitors, and mutant selection. Additionally, elicitation, discussed above in relation to improving bulk yields in cell culture, can also be used as a powerful tool to investigate pathway regulation based on gene expression. Plant cell cultures, including both suspension cultures and hairy root cultures, have proven to be an extremely useful platform for metabolic studies, as a fast growing and renewable source of material. Whole plants can also be valuable, particularly as models to study complex spatial and temporal control mechanisms associated with environmental stimuli and morphogenesis from a global metabolic perspective.[16]

Several approaches have been used to identify the enzymes and their corresponding genes which catalyze biosynthetic pathway steps. For the paclitaxel pathway, a successful approach utilized by the Croteau laboratory incorporated feeding cell free *Taxus* extracts with precursors to isolate and identify intermediate metabolites and enzymes. This approach led to the identification of taxadiene synthase, which catalyzes the first committed step of the taxane pathway.[83] Genes were subsequently identified from a cDNA library using PCR amplification based on degenerate primers designed to recognize conserved regions from homologous enzymes in other plants whose DNA sequences were known.[84] Differential display methods (via reverse transcription and PCR) comparing mRNA transcripts between elicited and unelicited cells supplemented with a homology-based search of a cDNA library from elicited cells,[85] as well as random sequencing of the same induced library,[86] have also proven to be extremely effective in gene discovery (for a comprehensive review of molecular genetics in *Taxus* see ref 87).

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An examination of the mRNA expression profile of known genes, either by Northern blotting or RT-PCR, can provide insight into pathway regulation. By comparing the time course of expression of 11 known genes in elicited and unelicited *Taxus* spp. cultures to total taxane accumulation, potential bottlenecks were identified based on low levels of mRNA transcript accumulation in MJ-elicited cells.[63] In this particular study, two late pathway steps were identified as likely rate influencing, and provide an objective for a targeted metabolic engineering approach by overexpressing these particular genes. In branched pathways such as the paclitaxel biosynthetic pathway, this type of analysis can also provide clues as to the predominate direction of flux.[63] Transcript profiling can also be used to evaluate the effect of differentiation conditions on gene expression. A rotation culture system was devised for *C. roseus* cultures in which cell lines were maintained as either suspension cultures or differentiated calli.[88] Analysis of mRNA levels of genes in the precursor pathways and secondary (terpene indole alkaloid or TIA) pathways, as well as activators and repressors, showed a reduction or loss of expression of several key TIA pathway genes and activators in proliferating suspension cultures.[88] These levels subsequently rose when differentiated cultures were reinitiated, confirming the close relationship between secondary biosynthetic pathway gene expression and cellular differentiation. In model systems such as *Arabidopsis* for which microarrays containing probes for thousands of genes are available, a more detailed transcript analysis has been performed to examine effects of nutrient stress conditions[89] and cell cycle progression.[90]

In order to implement a targeted metabolic engineering approach by overexpressing a pathway gene, a reliable transformation technology must be available to integrate foreign DNA into plant cells. A range of methods proven to work in plant species are available, and can be divided into two general classifications: *Agrobacterium*-mediated and direct gene transfer. *Agrobacterium* contains a Ti plasmid, a portion of which (T-DNA) is integrated into the plant genome after bacterial infection of the host cell. The T-DNA in wildtype bacteria contains genes to promote proliferation. Strains of bacteria where these tumor inducing genes have been replaced with genetic sequences of interest have allowed investigators to transfer genes by leveraging the native bacterial machinery.[91] *Agrobacterium*-mediated transformation has become the method of choice due to its low cost and ease of use, though many plant species, including commercially valuable species such as gymnosperms and cereal grains, are recalcitrant to this transformation method.[92] "Supervirulent" strains of *Agrobacterium* containing additional virulent proteins have been used to overcome this limitation (e.g., ref 93), and more recently, the manipulation of genes in the host plant associated with the transformation process has shown promise in improving the transformation efficiency of hard-to-transform plant species.[94] Host proteins involved in particular steps of the transformation process such as the initial host-bacteria contact, nuclear import of the T-complex, and integration, have been identified, and it may be possible to transport these proteins to the host cell using the same *Agrobacterium*, which can transport proteins independently of T-DNA.[94] Direct gene transfer techniques include polyethylene glycol (PEG)-mediated DNA uptake, silicon carbide fibers, electroporation, and microparticle bombardment. In particular, particle bombardment, in which micron-sized metal particles are coated with DNA and accelerated into the target cells at a sublethal velocity capable of penetrating the cell wall, is the transformation method of choice for those systems resistant to *Agrobacterium*.[91] *Agrobacterium*-mediated transformation has been successfully applied to a number of medicinal species, including *C. roseus*[95] and *A. annua*.[96] *Taxus* spp. systems lacked a reliable transformation method until recent reports of a stable *Agrobacterium*-mediated transformation[97] and a transient particle bombardment transformation developed by our laboratory.[98]

Metabolic Engineering Strategies

Biosynthetic pathways can be divided into two major stages: pathways common to most plants, which produce universal

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Table 2. Natural Product Biosynthesis in Native Species: A Comparison of Secondary Pathways

secondary metabolite	species	application	current commercial production method	metabolite classification	genes identified in secondary pathway	expressed sequence tags	regulatory elements identified
artemisinin	*Artemisia annua*	antimalarial	plant	sesquiterpene lactone	3 of >7[15]	52[a]	no
camptothecin	*Camptotheca acuminata*	antitumor	plant	monoterpenoid indole alkaloid (TIA)	4 of >11[7]	N/A	no
paclitaxel	*Taxus* spp.	antitumor	suspension cell culture and semisynthesis	diterpenoid	14 of 20[87]	22[a]	no
vincristine	*Catharanthus roseus*	antitumor	plant	monoterpenoid indole alkaloid (TIA)	10 of 18[4]	9042[a]	yes

[a] http://www.ncbi.nlm.nih.gov/dbEST/.

precursors for a class of products (e.g., flavonoids, terpenoids), and divergent pathways for specific products within a particular class (e.g., terpenoid subclasses taxanes and carotenoids). While both of these pathways are targets for metabolic engineering, much more is known about precursor pathways, since they are universal and have been studied in multiple different systems. For instance, all terpenoids are derived from the precursor isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). These precursors are supplied via two pathways: the plastidial 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway, named after the first committed precursor, MEP (also commonly referred to as the deoxyxylulose-5-phosphate or DXP pathway), and the cytosolic mevalonate (MVA) pathway. All of the genes in both of these pathways have been identified.[99] In contrast, the biosynthetic pathway from IPP to paclitaxel consists of 20 putative steps, for which 14 genes have been cloned,[87] and no transcription factors identified. Various secondary pathways have been defined to different extents, and an overview of the relative knowledge regarding the biosynthetic pathways for four important pharmaceutically active natural products is shown in Table 2.

Provided a suitable genetic transformation method is available, overexpression of genes associated with precursor pathways, to increase available precursor pools, and secondary pathways are common strategies to increase end product accumulation. While precursor pathways are better defined in terms of individual pathway steps, there are often multiple precursor pathways (such as the MEP and MVA pathways), about which many aspects of global metabolism, such as flux between the pathways, are unknown. Inhibitors of these precursor pathways can be used to investigate the source of precursor supply. Recent studies in *A. annua* for production of artemisinin[100] and *Taxus* for production of paclitaxel[101] indicate that precursors are supplied via both pathways. This approach is limited in that it cannot track precursor exchange between compartments to which each pathway is localized, and so cannot determine the proportion supplied by each pathway. Labeling studies can provide more definitive evidence as to the origin of precursor supply, and have been applied to show that salvinorin A is derived from the MVA pathway in *Salvia divinorum*,[102] and that gaudichaudianic acid is derived from both the MVA and MEP pathways in *Piper gaudichaudianum*, with transport of specific intermediates across the plastid boundary.[103] Overexpression of genes in precursor pathways has proven to be successful in increasing end products of secondary metabolism such as monoterpene essential oils in both peppermint[104] and lavender,[105] but has also been shown to have a limited impact on end products due to tight regulation of metabolite accumulation, as evidenced by a substantial improvement of intermediates but limited accumulation of alkaloid end products in *C. roseus*.[106] The direct overexpression of genes associated with dedicated secondary pathways has shown to be more effective in increasing alkaloid accumulation in *C. roseus*,[107] though the effect appears to be temporary, most

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likely as a result of the same factors which induce variability in nontransgenic plants (see above).

Transcription factors are difficult to identify in nonmodel species, but can be a more powerful tool to control metabolic flux since they can regulate multiple steps, and also offer the potential to regulate steps for which the component enzymes are unknown.[108] A classical genetics approach, which does not require information about genes or metabolic intermediates, has proven to be useful in nonmodel species for which this information is still only partially known. T-DNA can be used as an activator causing overexpression of genes flanking the insert, which results in dominant gain-of-function mutations.[109] Mutant phenotypes can then be screened based on properties such as pigmentation or resistance to a toxic metabolic derivative. This approach was successful in identifying the octadecanoid-derivative-responsive *Catharanthus* APETALA2 (AP2)-domain3 (ORCA3) transcription factor in *C. roseus* cells,[110] and subsequent overexpression of this gene upregulated several genes involved in TIA biosynthesis and increased overall accumulation of TIAs. The lack of prerequisite tools for this approach, such as a reliable genetic transformation method, has limited its application to other systems, such as *Taxus*, to this point.

Future Directions in Engineering Supply

Significant advancements have been made in understanding metabolite production dynamics in plant cell cultures on a number of levels, from bulk culture analysis and process design, to the recognition of subpopulations and heterogeneity at the single cell level, to metabolic pathways and their regulation within the single cell. An integrated approach within each level of analysis and relating all three levels will be needed to fully exploit the potential of plant cell culture systems to reliably and consistently supply metabolites at a sufficient yield to make these systems economically viable.

At the metabolic level, efforts would be enhanced by a system-wide analysis of plants, both in differentiated and undifferentiated tissues. The lack of complete information about the genomes of most medicinal plants—only three plant genomes (rice, maize, and *A. thaliana*) have been fully sequenced—make traditional "omics" approaches difficult to apply to these systems. The information needed to annotate plant genes solely based on sequence homology to genes of known or predicted functions in other systems is simply not available in many cases. The complexity and redundancy of many pathways coupled to incomplete knowledge of their regulation can lead to unpredictable results from a targeted metabolic engineering approach. Overexpression of phytoene synthase, the first committed enzyme of carotenoid synthesis in tomato, led to growth defects due to flux diversion from gyberrillin synthesis.[111] This demonstrates the importance of understanding the implications of increased flux through one step of a pathway on the entire system. Transgenic manipulation of pathway enzymes has proven to be unstable, as gene expression and alkaloid accumulation eventually returned to the level of nontransgenic cultures in *C. roseus*,[112] which may result from regulatory factors further downstream of the targeted genes. Transcription factors are more likely to be effective in controlling flux through a specific pathway or branch; however it is often necessary to increase precursor availability using this approach, and a complete understanding of the coordination of multiple branches and sections of metabolic pathways is likely to require an integrated metabolic model including information from genomics, transcriptomics, proteomics, and metabolomics. A system wide analysis of metabolic flux can be quantified through labeling experiments,[113] which facilitates the construction of predictive models. Integrated systems biology approaches including genome-wide functional genomics models have been largely limited to model plant systems, though recent progress has been made in developing gene-to-gene and gene-to-metabolite networks in *C. roseus*.[114]

An integrated approach will most likely be necessary in successful engineering efforts to reliably increase metabolite production. For instance, as many secondary metabolites are toxic due to their native biological functions, artificially high induced levels of production via metabolic engineering may be detrimental to primary cell function, but may be mitigated in a product removal or cell encapsulation scheme. Population dynamics within a culture due to environmental effects may also prove to be the decisive factor in metabolite accumulation, as manipulation of secondary pathways may only affect a small subpopulation of cells. Despite the complexities of plant metabolism and open questions regarding population dynamics, substantial progress has been made in characterizing and utilizing native medicinal plant species for the production of valuable natural products. Although paclitaxel is a notable commercial success, plant cell culture is still an emerging technology. Continued advancements in

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the understanding of secondary metabolism within cells and within cell cultures are critical to eventual viability of plant cell culture as a means to supply vital natural products, which may have no alternate means of supply.

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