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Standard Review

The development of the pitcher plant *Sarracenia purpurea* into a potentially valuable recombinant protein production system

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The unique inducible system of protein secretion by the carnivorous pitcher plant *Sarracenia purpurea* may be an ideal system for recombinant protein farming. *S. purpurea* is relatively uncommon and difficult to grow *in vitro*, so it has not been explored as a potential source of recombinant proteins. However, it naturally secretes large amounts of proteins into a liquid found in the leaf pitchers, so it may be an ideal way to collect recombinant proteins in leaf pitchers. Here, the advantages of transgenic *S. purpurea* systems over traditional transgenic plant systems for the production of recombinant pharmaceutical proteins are explored, and the steps necessary to produce such a system are discussed.

Key words: Transgenic plants, recombinant protein farming, carnivorous plants, gene technology.

INTRODUCTION

The purple pitcher plant *Sarracenia purpurea* is a carnivorous rosette-forming plant that grows in sphagnum bogs, nutrient poor fens, seepage swamps, and pine savannas of the eastern United States and Canada (Ellison et al., 2004; Schnell, 2002). Many pitcher plants can be found in the region surrounding Lakehead University in Thunder Bay, Ontario, Canada, where some research on this plant is taking place. *S. purpurea* has modified pitcher-shaped leaves which serve as reservoirs to collect rainwater, into which the plant secretes its own liquid, as well as hydrolases and other proteins for prey digestion (Figure 1).

Secretions are produced at the lip (or “hood”) of the leaves, which attract prey to the plant. Prey attracted to the plant are directed downward by hair-like spines on the inside of the hood, and eventually contact and drown in the liquid contained in the pitchers (the “trap”) (Ellison and Gotelli, 2002). There, prey is digested by a number of hydrolases including RNases, nucleases, phosphatases, and proteases (Gallie and Chang, 1997). The trapping mechanism in *S. purpurea* is so efficient that higher animals such as frogs are often found partially digested inside the pitchers, which is why the plant is classified as carnivorous rather than insectivorous (Lindquist, 1975). There has been considerable debate over whether digestive enzymes in the pitcher are produced by microorganisms living in the plants or by the plants themselves, but many studies indicate that microorganisms may just play an incidental role in prey digestion, and instead play a critical role only in nitrogen acquisition and fixation (Bradshaw and Creelman, 1984; Gallie and Chang, 1997). Some studies state that *S. purpurea* relies on bacteria for essential digestive processes, but these studies present no data from enzymatic tests on sterile pitcher fluid, and contain no references that do (Adams and Smith, 1977; Harvey and Miller, 1996; Heard, 1994). In addition, nitrogen demand by the plant strongly affects the expression of carnivory, and the pitchers continue to accumulate digestive enzymes even in the absence of any microrganisms (Ellison and Gotelli, 2002; Gallie and Chang, 1997; Givnish et al., 1984).

CARNIVORY IN *SARRACENIA PURPUREA*

Carnivory in pitcher plants is thought to have evolved primarily for nitrogen acquisition, as they typically grow on nitrogen-poor peat bogs and cease expression of carnivory in nitrogen rich environments (Ellison and Gotelli,

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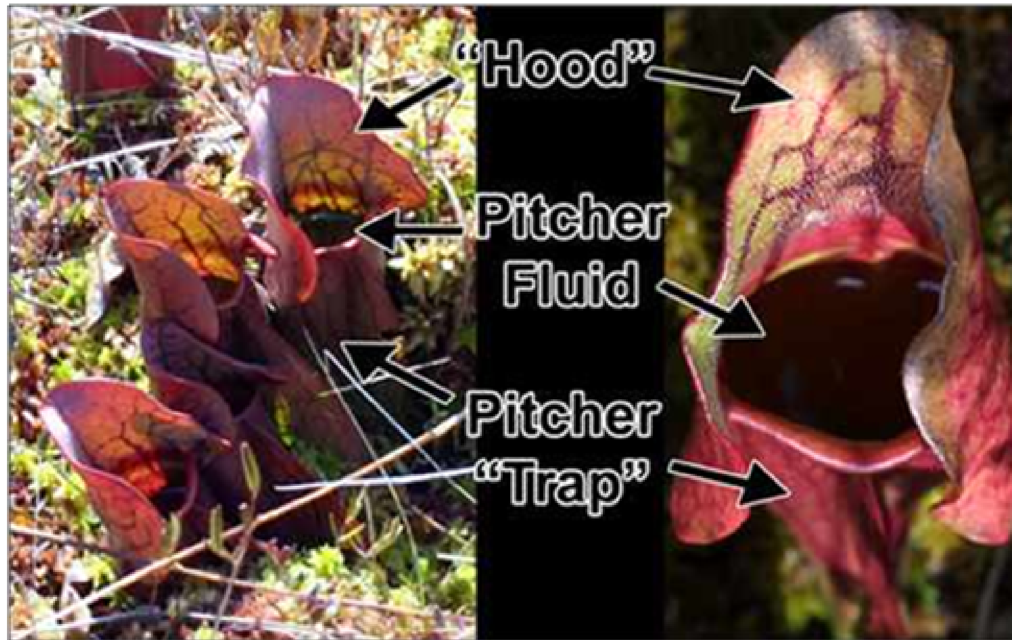

Figure 1. The basic anatomy of the North American pitcher plant *Sarracenia purpurea*. Pictures were taken by Bruce Rosa at William's Bog, near Kingfisher Lake in Thunder Bay, Ontario, Canada.

2002; Givnish et al., 1984). Phosphate and potassium acquisition is also primarily achieved through carnivory (Chapin and Pastor, 1995; Christensen, 1996; Jaffe et al., 1992).

Upon first opening, the traps secrete and accumulate hydrolases for approximately one week, after which time hydrolase secretion reduces to low levels until the trap is induced by prey (Gallie and Chang 1997). RNase, nuclease and protease activity all appear to be induced by the presence of either protein or nucleic acids, suggesting a coordinated inducible response to prey for these hydrolases (Gallie and Chang, 1997). However, phosphatase secretion is only induced by proteins and not nucleic acids, suggesting additional complexity to the induced response (Gallie and Chang 1997). Mechanical stimulation of the plants has no effect on prey recognition and the subsequent release of hydrolases, as it does in other carnivorous plants such as the Venus' flytrap *Dionaea muscipula* (Robins, 1976). Further study is necessary in order to understand the complex pathways in the carnivory response of *S. purpurea*.

The ability to produce hydrolases only in the presence of prey significantly reduces the metabolic cost of carnivory to the plant. This is necessary because prey capture efficiency is commonly very low, with some studies reporting an average of 0.070 prey $leaf^{-1}$ day^{-1} (Newell and Nastase, 1998). The detection of prey in one leaf does not induce hydrolase secretion in another leaf on the same plant (Gallie and Chang, 1997). This trap-specific response is another apparent adaptation to reduce the metabolic cost of carnivory.

Other compromises are made by *S. purpurea* in order to balance the cost and the benefit of carnivory. It has been observed that the presence of a strong red coloration in the leaves, indicating the presence of nectar, serves as a strong attractant for prey at the cost of the energy required to produce the nectar (Cresswell, 1993). Other studies indicate that having larger traps increases the ability of the plants to successfully trap prey, at the cost of additional nitrogen spent to increase the size of the traps (Cresswell, 1993; Gibson, 1991). Perhaps because of these trade-offs, there is pronounced morphological variability in terms of the size, shape, and color of leaves between *S. purpurea* across its geographical range (Buckley et al., 2003; Schnell, 1979; Schnell, 2002). Even in one location, these characteristics can change dramatically in response to local environmental conditions, particularly temperature and moisture (Ellison and Gotelli, 2002; Mandossian, 1996). The pronounced variability in these visual characteristics in response to the pitcher plant's surroundings and prey suggests that the plants may also develop distinct hydrolase induction mechanisms and secretion responses. Little, however, is known about the exact amounts and specific types of all the hydrolases secreted by *S. purpurea*.

THE ROLE OF MICROORGANISMS IN PITCHER PLANT DIGESTION

The liquid in the trap of *S. purpurea* contains a suite of decomposers including bacteria, protists, rotifers, and fly larvae, which may aid in the digestive process, but likely only play an incidental role (Bradshaw and Creelman, 1984; Ellison and Gotelli, 2002; Gallie and Chang, 1997;

Harvey and Miller, 1996; Whitman et al., 2005). Immature pitchers are closed and sterile, but once opened and matured, the species complement of microorganisms present in them varies considerably even in the same geographical region, due to random introductions by insects and wind, competition between microorganisms and due to the differential availability of nutrients (Hepburn and St. John, 1927; Whitman et al., 2005). Microorganisms appear to be introduced to the pitcher most often by prey, which may bring bacteria from any outside source, or may cross contaminate pitchers when they successfully escape one trap and then enter another (Gibson, 1991; Whitman et al., 2005). The types of bacteria isolated from pitchers have been also been found to be very similar to the bacteria harbored in the complex microbial communities of the exoskeletons and guts of common prey insects (Siragusa et al., 2007).

It is unlikely that microorganisms play a critical role in prey digestion, as *S. purpurea* continues to produce hydrolytic enzymes after treatment with the antibiotics ampicillin, carbenicillin and cefotaxime at quantities sufficient to effectively inhibit all microbial activity (Gallie and Chang, 1997). Pitchers also secrete liquid containing high concentrations of hydrolases even in the absence of water and microorganisms in the pitcher (Gallie and Chang, 1997). It has also been suggested that the digestion of proteins by bacteria is too slow to be beneficial to the plant (Hepburn and St. John, 1927). Although microorganisms do not appear to play an important role in prey digestion, *S. purpurea* does appear to rely on bacteria for aiding in the acquisition and conversion of nitrogen sources, which is essential for the plant's growth (Butler et al., 2008; Harvey and Miller, 2006).

The microorganisms present in the traps of *S. purpurea* may be an interesting subject for further study, as any organism living inside the pitcher traps are somehow protected against the harsh hydrolytic environment created by the plants. This protection may be due to the development of cell membranes immune to degradation by hydrolases, or due to the presence of inhibitors of excreted proteases and hydrolases, which would neutralize threats from the pitcher's digestive processes.

The active protein excretory system in *S. purpurea* is a unique trait that may be exploited in order to design of a recombinant protein farming system that is more profitable than traditional transgenic plant systems.

Traditional transgenic plant systems

Production of recombinant proteins in transgenic plants and plant cell cultures results in high quality proteins that are generally safer for use as drugs than proteins harvested from bacterial or mammalian sources due to the low risk of contamination by mammalian viruses, pathogens and toxins (Table 1) (Ferrante and Simpson, 2001; Ma et al., 2003).

In whole-plant systems, proteolytic activity within transgenic plant cells adversely affects recombinant protein yields (Ma et al., 2003). Subcellular targeting of recombinant proteins in plant systems is a common method of isolating the proteins from proteases without inhibiting the critical functions of these proteases in the plants (Ma et al., 2003). Even in these systems, however, it is difficult to obtain high yields of recombinant proteins.

Targeting recombinant proteins to the cytoplasm is inefficient because it exposes proteins to 20S and 26S proteasomes which rapidly degrade the protein with the help of ATP hydrolysis via the ubiquitination system (Callis, 1995). The plant cell vacuole contains serine, cysteine and aspartic acid proteases, metalloproteinases and other unidentified proteases which degrade proteins rapidly despite the vacuole's capacity for storage space (Callis, 1995; Hara-Nishimura et al., 1991). The chloroplast lacks the ubiquitin system and produces high levels of protein, but contains other proteases, some of which are similar to prokaryotic proteases and some of which are transported into the chloroplast from the cytoplasm (Adam, 1996; Malek et al., 1984; Watson et al., 2004).

One of the more efficient methods for recombinant protein production is targeting proteins for retention in the endoplasmic reticulum. This is an effective strategy due to its oxidizing environment, the presence of molecular chaperones and a lack of proteases (Ma et al., 2003). Novel cellular targets have emerged recently, including targeting recombinant proteins to the oil bodies of seeds, which are relatively easy to purify from the other proteins in the plant cell (Kiihnel et al., 1996; Nykiforuk et al., 2006). However, despite the fact that these systems manage to produce relatively high yields of properly folded proteins, all present transgenic whole-plant protein harvesting systems require destruction of the plants or seeds, which results in the need to wait for plant growth, and to pay for growing materials.

Plant cell culture secretion systems circumvent the typical expensive extraction process because purification is performed on the liquid medium instead of cell lysate. Excreted recombinant proteins have been shown to fold more efficiently than proteins targeted intracellularly, resulting in more active forms of recombinant proteins that degrade less due to a lack of proteases (Schillberg et al., 1999). Purification of the liquid medium is easier because there is much lower protein contamination from cellular components, and intracellular proteases are not exposed to the foreign protein. However, purification is complicated by the severe dilution of the target protein. The total soluble protein content of the recombinant protein is highly variable in plant cell culture systems, but is often as low as 0.05% (Doran, 2000; Hellwig et al., 2004). In addition to producing low concentrations of recombinant proteins, production and maintenance of plant cell culture systems is expensive, so commercial protein farming companies typi-cally utilize whole-plant systems instead.

Table 1. Comparison of production systems for recombinant pharmaceutical proteins, adapted from Ma et al., 2003.

System	Overall cost	Scale-up capacity	Production timescale	Product quality	Glycosylation	Contamination risks
Transgenic Plants	Very Low	Very High	Long	High	Minor Differences	Low Risk
Plant Cell Cultures	Medium	Medium	Medium	High	Minor Differences	Low Risk
Transgenic *S. purpurea* [a]	Low	High	Short [b]	Unknown	Unknown	Low Risk
Bacteria	Low	High	Short	Low	None	Endotoxins
Yeast	Medium	High	Medium	Medium	Incorrect	Low Risk
Mammalian Cell Culture	High	Very Low	Long	Very High	Correct	Viruses, Prions and Oncogenic DNA
Transgenic Animals	High	Low	Very Long	Very High	Correct	Viruses, Prions and Oncogenic DNA

[a] - Projected.
[b]-The initial production of the system would take a long time, but once developed, protein production would be relatively fast because the plants would not need to be destroyed to harvest the proteins.

Newer and more efficient transgenic plant technology has allowed companies to patent and produce more sophisticated and profitable recombinant protein systems. Recombinant pharmaceutical antibody production plants have been developed for commercial use from transgenic tobacco, alfalfa, corn, rice and other crops (Stoger et al., 2002). Sigma Inc, a chemical and molecular bio-logy supply company, has been selling recombinant β-glucuronidase derived from the cytoplasm of transgenic corn seeds for several years (Ma et al., 2003). Using newer technology, Sembiosys Inc has produced transgenic safflower seeds which produce insulin bound to oil bodies (Stephan, 2008).

These recombinant proteins are more easily purified from the rest of the seed than recombinant proteins expressed in seed cytoplasm (Nykiforuk et al., 2006; Stephan, 2008). The purified insulin product is expected to be commercially available by 2012 (Stephan, 2008).

In most intracellular transgenic plant protein systems, protein expression is limited by post-transcriptional gene silencing (PTGS). Viral-based transient expression vector systems such as tomato bushy stunt virus can prevent the onset of PTGS, resulting in higher levels of active intracellular protein expression (Voinnet et al., 2003).

Transgenic rhizosecretion systems, in which tobacco plant roots secrete recombinant antibodies which can later be harvested, are under development and have the potential to become profitable biotechnology systems (Drake et al., 2003). These secretory systems have the advantage of producing relatively pure recombinant proteins, without the difficulty of maintaining cell cultures (Drake et al., 2003).

TRANSGENIC *SARRACENIA PURPUREA* SYSTEMS

Aseptically grown *S. purpurea* has the potential to be developed into a novel transgenic whole-plant system in which recombinant proteins are secreted into sterile liquid in the pitcher. This system would circumvent the need to destroy the plant or the seeds, as is currently required for whole-plant recombinant protein systems. Because *S. purpurea* is naturally adapted to secrete high levels of com-plex proteins, it has the potential to produce much higher total yields of recombinant proteins than rhizosecretion systems. As well, *S. pur-purea*'s specialized secretory cells may be a uni-que target for developing cell suspension systems that produce much higher total soluble protein content than traditional plant cell suspension cultures.

The development of a transgenic *S. purpurea* system

The hydrolytic enzymes secreted by pitcher plants must be identified and quantified in order to identify genes with high expression levels in secretory pathways. Using a gene vector method such as *Agrobacterium tumefaciens*, these target genes could theoretically be replaced with genes for commercially important recombinant proteins such as insulin, interferon or kinase C. However, genetic transformation systems have not yet been tested in *S. purpurea.*

Proteolytic enzymes secreted in the pitchers will be responsible for some degradation of the target

recombinant proteins in this proposed system unless they are inactivated. It is not currently known how many specific proteases are produced by the pitchers of *S. purpurea*. However, once identified, these genes could be knocked out using gene technology such as transposon muta-genesis, TILLING (targeted, induced local lesions in genomes), which uses chemicals targeted for specific gene sequences, or Deleteagene, which induces deletion mutations using fast neutrons (Feng, 2006). These genes could also be inhibited by the use of specific enzyme inhibitors, but these would reduce the economic feasibility of the experiments, as they are relatively expensive and would need to be continually added to the pitcher fluid to ensure complete inhibition. It is not currently known how many specific proteases are produced by the pitchers of *S. purpurea*.

It is unclear how stable recombinant proteins in pitcher fluid will be, even in the absence of proteases. However, pH-adjusted sterile buffers optimized for each recombinant protein could theoretically be utilized to ensure protein stability without inhibiting secretion rates.

Since carnivory is only expressed in pitcher plants that are low on nitrogen, completely eliminating protease activity in the medium could be harmless to the plant if nitrogen and phosphate (in the form of NH_4NO_3) is added after harvesting the recombinant protein (Ellison and Gotelli, 2002). This is far different from traditional whole-plant systems, in which protein expression is intracellular and proteolytic activity cannot be eliminated because proteases are required in many cellular pathways (Bond and Butler, 1987).

High rates of recombinant protein secretion by the plants could be induced by nitrogen starvation and by signaling from target proteins and nucleotides in the pitcher (Ellison and Gotelli, 2002; Gallie and Chang, 1997). In order to optimize the protein secretion rates it will be necessary to identify the specific amino acid or nucleotide sequences that result in the highest secretion rates in the plants. Optimal levels of nitrogen supplementation would also need to be identified, as nitrogen would be depleted to create the recombinant proteins, but supplementing too much nitrogen would result in a loss of carnivory.

The overall design for a transgenic whole-plant *S. purpurea* system for recombinant protein farming would therefore involve supplementing the transgenic pitchers (containing the recombinant gene and knocked out secretory enzyme genes) with sterile water containing the inducer molecule and small amounts of nitrogen, then collecting the liquid after a period of time and purifying the relatively pure secreted recombinant protein from it.

Once optimum induction and secretion rates are identified, a continuous harvesting system should be developed in order to quickly purify recombinant proteins, which would ensure minimal degradation.

S. purpurea cell suspension cultures should also be considered for use in a recombinant protein harvesting system. Secretory cells from the inside of the pitcher could be isolated and cultured in order to develop a novel cell suspension system, in which the cells are inherently programmed for high rates of protein secretion. These cultures may produce much higher overall recombinant protein yields than traditional plant cell suspension cultures. Once developed, this system would also circumvent the difficulty of growing whole *S. purpurea* plants aseptically.

CONCLUSION AND DISCUSSION

Current literature on the topic of enzyme secretion in *S. purpurea* is complicated by the presence of a variety of microorganisms found in the natural environment. Growing *S. purpurea in vitro* and in sterile conditions is difficult, and has contributed to the lack of knowledge about the molecular pathways inside the pitchers. However, *S. purpurea* has the potential to become a profitable transgenic plant system for recombinant protein production. It has a unique natural system of protein secretion that may be utilized to develop cell suspension systems with high recombinant protein yields, or whole-plant systems in which no tissues or seeds need to be destroyed in order to harvest the recombinant protein. This whole-plant system would reduce costs associated with growing the plants as well as with purifying the target protein, so it may present a more profitable and commercially successful alternative to current transgenic systems.

A significant amount of research will have to be performed in order to identify the enzymes that are secreted at high rates in response to the presence of prey in the pitchers, which would serve as targets for recombinant protein genes. The actual amount of any specific enzyme secreted by the plants is not known at this time, but this measurement would be relatively simple and would help to determine the economic feasibility of a transgenic whole-plant *S. purpurea* system. The remaining secreted enzymes and the gene regulatory pathways leading to specific hydrolase induction and excretion would also need to be characterized and then eliminated by gene knockout technology. This research could also potentially lead to the discovery of novel enzymes secreted by these relatively poorly known plants.

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