Chapter 1

Introduction: Biology of orchids and of orchid mycorrhizal interactions

With the nursery and garden center industry expanding, it is important to keep the consumer returning year after year for the newest, most exciting and most unusual species. Perennial nursery crops are one sector of the nursery market that has grown rapidly due to the addition of new species. Though orchids are not new to the horticultural trade, these fascinating plants have long been in the domain of the specialist. With interest in native plants growing, the propagation and sale of our native orchids will bring these plants to an expanding market. The potential for orchids is large as the native species offer the exotic blooms of the tropical species with superior hardiness. Even the smaller flowered species are comparable to the small-flowered spring bulbs beloved by many. Native orchids will add much-demanded new crops to the horticultural trade and substantially boost returns for propagators, growers and sellers. The objective of this research is to develop new techniques that will significantly improve the *in vitro* germination of native orchid species. This will be accomplished by the use of molecular biological methods to identify genes expressed during mycorrhizal infection of germinating orchid seeds. The characterization of these genes through computer biology or through *in vitro* studies will increase understanding of one example of orchid-mycorrhizal symbiosis.

Orchid Biology

The Orchidaceae is one of the largest plant families with close to 35,000 species and a circum-global distribution (Dressler, 1993). It is also one of the most advanced plant families with many adaptations enabling long-term survival. One adaptive mechanism is that the roots of many orchids, especially tropical species have a multiple epidermis of dead cells, called velamen, that protects the root cortex from excessive drying and aids in water uptake (Pridgeon, 1987). Another example can be seen in orchid flowers which have evolved to have a close relationship with insect pollinators such that sympatric species (species growing in close proximity that have the potential to interbreed and form hybrids) can each be pollinated by a separate insect species (Van der Pijl and Dodson, 1966), maintaining species isolation (necessary to prevent interbreeding of species). A reliance on mycorrhizal interactions is a third adaptive mechanism that has allowed orchids to persist in less than ideal habitats and has led to their occurrence worldwide.

Along with root velamen, the symbiotic relationship of primarily terrestrial orchids with soil fungi has allowed these plants to adapt to habitats as diverse as arctic tundra and rainforest canopies. Benzing (1987) states that these mechanisms aided the colonization of the epiphytic realm, which led to large and rapid species radiation. Mycorrhizal interactions also allow temperate orchids to persist for many years in a dormant state as the plant feeds on its fungal symbiont (Arditti, 1992). In the most

derived form of this interaction, some orchids have become wholly parasitic on fungi and do not produce chlorophyll (e.g. *Corallorhiza;* Arditti, 1992).

Another aspect of this mycorrhizal interaction is the development of microspermy, which means small seed and refers to the tiny, underdeveloped orchid seed (Benzing, 1987). Microspermy is believed to have aided in the development of the all or nothing event associated with orchid pollination. Pollen in the Orchidaceae is delivered *en masse* as a pollinium (pollinia), a structure in which the pollen within an anther is fused into a mass (Arditti, 1992). If an insect fails to deliver this mass of pollen, or no insect pollinators are attracted to the flower, the pollen is not delivered and there is no fruit set. If the pollinia are deposited on the stigma, all the pollen is delivered at once and can potentially fertilize every ovule. One pollination event leads to the production of hundreds of thousands of seeds (Benzing, 1987).

Microspermy is energetically inexpensive. At pollination ovules are not developed and only three placental ridges can be seen in the ovary; however, some species do have ovules in various stages of development (Yeung and Law, 1997). After pollination, ovule development proceeds so that fertilization can take place. Double fertilization is undertaken as in other angiosperms, however the endosperm fails to develop. Even after fertilization, the zygote may rest for some time before division and embryo development. At maturity, the seeds contain little more than a rudimentary embryo and no endosperm (Arditti, 1992). This helps to ensure the survival of the parent plant since little energy is channeled to both the unpollinated ovary and the thousands of fertilized ovules. However, the seeds have no stored nutrients for subsequent growth and cannot survive without a mycorrhizal interaction. The adaptive mechanism that has allowed orchids to colonize diverse habitats and to persist in a semi-dormant state can also prevent seed germination and seedling growth.

The orchid seed consists of a testa surrounding a tiny embryo in the globular stage with no defined cellular organization suggestive of embryonic tissue found in other angiosperms (Arditti, 1992). The chalazal end of the embryo develops into the shoot apex and roots arise from other parts (Arditti, 1979). Upon germination, the embryo swells to form a protocorm that will develop rhizoids (Arditti, 1992). After a few days (or several weeks) the protocorm develops a shoot apex with leaf primordium and a root will form. During the early stages of growth prior to shoot apex formation, cell division takes place in the chalazal end of the embryo while cells enlarge but show little or no division at the micropylar end (Vellarpillai et al., 1997). This is combined with DNA synthesis; however, micropylar cells do not divide following DNA replication suggesting endoreduplication (Raghaven and Goh, 1994). Levels of mRNA also increase especially at the chalazal end of the embryo. These results indicate changes in gene transcription in association with germination though changes in gene transcription are not related to fungal infection.

Seed Germination

Most orchid seeds cannot germinate naturally in the absence of mycorrhiza. Noel Bernard discovered the role of mycorrhiza in seed germination in the late 1800s and Hans Buergeff worked on orchid mycorrhiza in the early part of last century (Arditti, 1992). Their work showed that orchid mycorrhizal interactions were fairly specific and orchid

seeds would not germinate without a fungal symbiont. It is now known that though some species do have specific interactions with certain species of fungi, others have a general relationship with many species of fungi (Arditti et al., 1990). It is also known that orchids can be germinated without their fungal symbiont as Knudson (1922) found that orchids could be germinated asymbiotically on special media. Most orchids are now grown in this manner and many clones are cultured from meristems. The Knudson medium has undergone some changes to provide for different species and different methods of culturing orchids.

Asymbiotic seed germination has become the favored method for orchid production. Most tropical epiphytes are produced in this way (Arditti et al., 1990; Arditti et al., 1982). However, attempts to germinate terrestrial orchids asymbiotically have not been as successful and only a few species have been germinated asymbiotically (Arditti and Oliva, 1981; Oliva and Arditti, 1984). Symbiotic seed germination of temperate terrestrials is more effective as these orchids show a strong dependence on mycorrhizal fungi. However, symbiotic seed germination can be difficult to control and is fairly complicated (Stoutamire, 1974). Zettler (1997) reviewed the state of symbiotic seed germination and suggested that this technique was important for those taxa which do not respond to asymbiotic methods.

Symbiotic germination of terrestrial orchids has been used as an alternative to asymbiotic methods with some success. Clements (1986) found that species of *Orchis* and *Dactylorhiza* germinated better in the presence of fungi though not all seeds germinated nor did seedlings develop. Smreciu and Currah (1989) found the same for North American and European species. Several species in their study, including two *Corallorhiza*, failed to germinate; *Cypripedium calceolus* L. and *Calypso bulbosa* (L.) Oakes had low germination percentages in asymbiotic medium only. Zettler (1997) germinated several species with good results, though soil establishment was poor and some species did not germinate (*Isotria medeoloides* (Pursh.) Raf. and *Corallorhiza odontorhiza* (Willd.) Nut. Work with *Spiranthes magnicamporum* Sheviak (Anderson, 1997) and *Platanthera clavellata* (Mich.) Luer. (Zettler, 1998) showed that while asymbiotic media effected germination, growth and transplant survival were best if seed was inoculated with a symbiont. Knudson (1922), in the course of proving that orchids could be germinated asymbiotically, found that contaminating fungi and a species of bacteria both improved the growth of protocorms. It is important to note that though initial stages of germination (water uptake and rupturing of the testa) can occur without a symbiont, the completed process and subsequent seedling growth require infection and thus this technique is referred to as symbiotic germination.

Work with symbiotic germination implies that the fungal interaction is not highly specific, though Arditti et al. (1990) suggest that it may be in some species. Zettler and McInnis (1993) found that the mycorrhizae isolated from a species of *Platanthera* promoted germination of several North American orchids. However, *Isotria medeoloides* failed to germinate *in vitro* with fungal isolates and in the field with naturally occurring fungi. Smreciu and Currah (1989) also found that though 15 species of North American and European orchids responded to different fungi, several fungal isolates induced germination in more than one species of orchid. They also showed that *Ceratobasidium cereale* Murray and Burpee could become pathogenic on North American species. Though germination was stimulated *in vitro*, field transplant survival was low suggesting

that the fungus used to germinate seeds *in vitro* may not be suitable for growth *in situ*. Masuhare and Katsuga (1994) showed that seeds germinated in a turf grassland were infected by a single species of *Rhizoctonia* even though field isolates of other *Rhizoctonia* species were able to induce germination *in vitro*. Further, Knudson (1922) found that species of *Penicillium*, which are not considered orchid endophytes (see below), were pathogenic, but could also stimulate growth.

Symbiotic seed germination has proven useful in the propagation of terrestrial orchids from Australia (Clements, 1981; Clements, 1982; Clements and Elyard, 1979), Europe (Clements et al., 1986; Smrecieu and Currah, 1989) and North America (Zettler, 1994; Zettler and McInnis, 1993). However, it is not always efficacious and has not been successful in all species tested (Zettler, 1997). Arditti et al. state that "even with the best methods, germination [of temperate terrestrials] is relatively poor and not easily reproducible."

Factors such as light and temperature can also affect orchid germination. Zettler and McInnis (1994) found that light increased the symbiotic seed germination of *Platanthera integrilabia* (Correll) Luer. The same was found for the European species *Dactylorhiza majalis* (Rchb. F.) P. Hunt and Summesh (Rasmussen and Rasmussen, 1991). However, Arditti et al. (1990) stated that "light may inhibit the germination of some temperate terrestrials," as Stoutamire (1974) found with seeds of *Cypripedium* species. Temperature effects on germination are unclear as different species show different responses to cold and warm temperatures (Stoutamire, 1974; Ichihashi, 1989). Zettler and McInnis (1993) found that *Spiranthes cernua* (L.) Rich. responded favorably to chilling storage while *Goodyera pubescens* (Willd.) R. Br. did not. Rasmussen (1992) showed that a warm incubation followed by cold storage promoted germination of *Epipactis palustris* (L.) Crante. Abscisic acid (ABA) has been found in orchid seeds (Van der Kinderen, 1989) though hormonal applications that break ABA induced dormancy do not affect orchid seed germination (Reviewed in Arditti et al., 1990). Of interest, however, are the results of a study by Wilkinson et al. (1994) that showed that IAA enhanced symbiotic germination of an Australian terrestrial orchid. They believed that bacteria associated with orchids were the source of IAA *in situ*.

Mycorrhizal interactions

The relationship of orchids with fungi is relatively unique in the plant kingdom. Of the greater than 80 percent of plant species that form mycorrhizal relationships, orchids along with members of the order Ericales do not form endomycorrhizal relationships with genera of the Zygomycota. The main group of fungi inhabiting orchid roots is Basidiomycetes, though Ascomycetes have been found (Currah et al., 1997). Some of the Basidiomycetes with which orchids form a relationship are pathogenic on other crops, e.g., *Rhizoctonia solani* Kahn (Hadley, 1982). Even within the Orchidaceae, symbiotic fungi of one orchid species may be pathogenic on another. However, most orchids are able to control the infection and growth of endomycorrhizal fungi.

Orchid mycorrhizal fungi are found intracellularly in cells of the cortex and they are confined to roots (Hadley, 1982). Infection is limited to suspensor cells of the embryo and epidermal hairs and is highly restricted compared to fungal infection of non-orchid species. This suggests that orchids control the infection process, and that fungal

symbionts are adapted to this control (Hadley, 1982). Within the cells, the mycorrhizae form dense coils of mycelium called pelotons which are thought to be adaptations to the host cell (Hadley, 1982). Within the cell, the pelotons are surrounded by a membrane and interfacial matrix material (Peterson et al., 1997). The membrane lacks adenylate cyclase activity but is otherwise similar to the plasma membrane. The orientation of microtubules and cell wall microfibrils is altered during infection and may be necessary to alteration in the cytoplasm and synthesis of the membrane surrounding the pelotons (Peterson et al., 1997).

Other factors of the orchid-mycorrhizal interaction are also important to our knowledge of symbiotic seed germination. Hadley (1982) reviews several studies that show how terrestrial plants grown with a symbiont have greater growth compared to plants grown asymbiotically. Anderson (1991) shows that *Spiranthes magnicamporum* had greater growth when grown with a fungal symbiont than without. Hadley (1982) also reviews the process of nutrient exchange between plant and fungus (see also Arditti et al., 1990). It has been shown that vitamins, amino acids and sugars are translocated from the fungus to the orchid. However, it is not known whether these compounds are translocated across a living interface or released upon digestion of the fungus (Hadley, 1982). Purves and Hadley (1976) show that starch, accumulated during asymbiotic culture, is rapidly broken down upon infection and seeds germinated in the presence of an endophyte do not accumulate starch. Arditti et al. (1990) state that orchids do digest their fungal symbiont and Hadley (1982) notes that, though digestion is the ultimate end for endomycorrhizal fungi, it is not known whether the enzymes for digestion are produced by the plant or the fungus. Blakeman et al. (1976) showed that the activity of peroxidase, ascorbic acid oxidase, polyphenol oxidase and catalase increased upon mycorrhizal infection and that this was in part due to digestion of the fungus. It has even been suggested that mycotrophy, the digestion of mycorrhizal fungi within infected seedlings can increase the water content of infected seedlings (Yoder et al., 2000).

Hadley (1982) points out that orchids control the level of infection and that phytoalexins appear to mediate this control. The level of this control may relate to the orchid-fungus specificity of some species and the pathogenecity of some fungi in other orchid species. Also, it implies a moderate defense response by the orchid. Beyrle et al. (1995) found orhcinol in protocorms of *Orchis*. This was surprising as orchinol is a phytoalexin (the first discovered) and believed to inhibit fungal growth. Fungi readily infect protocorms so orchinol would not be expected to be present; however, its presence suggests expression of defense genes albeit at a level that does not interfere with infection. That mycorrhiza are not found in mature pseudobulbs or tubers (Hadley, 1982) supports the expression of defense genes at some point during mycorrhizal infection. Blakeman et al. (1976) hypothesized that an increase in oxidative enzyme activity upon mycorrhizal infection was similar to the oxidative activity of plants resistant to pathogens again indicates a defense response on the part of the orchid.

The preceding discussion highlights some of the physiological processes that are involved with mycorrhizal formation in orchids; however, it reveals much that is unknown. Inferences can be made about some of the events that lead to mycorrhizal formation in orchids from looking at other types of mycorrhiza. In endomycorrhiza, flavonoids and phenolic compounds have been suggested as signal molecules released by the plant that induce spore germination, pre-infection hyphal growth, and branching of

Glomus species (Hirsch and Kapulnik, 1998). These compounds are possibly effective in directing growth of ectomycorrhizal mycelium to a suitable host. Once fungal mycelium contacts the host root, specific cell-cell interactions lead to the formation of mycorrhiza (Hirsch and Kapulnik, 1998; Martin et al., 1999). These cell-cell interactions are host specific such that interaction of a fungus with a non-host will not lead to formation of structures characteristic of myccorhizal infection.

The plant fungus interface represents one of the earliest stages of mycorrhizal formation and as such would reveal much about the physiological processes involved with mycorrhizal formation. In ectomycorrhiza, several wall associated proteins show changes in expression when the fungus interacts with the host root (Martin et al., 1999). Glycoproteins consisting of N-linked mannose residues seem to be involved in recognition and early adhesion events. Another group of proteins, the hydrophobins, which make the fungal cell wall hydrophobic, also seem to be involved in fungal adhesion to the host. The most interesting group is the symbiosis-regulated acidic proteins (SRAPs). These proteins are up-regulated after adhesion during Hartig net and mantle formation. They also contain an RGD (Arg – Gly – Asp) motif which has been shown to interact with fibronectin- and integrin-like proteins. Since fibronectin and integrin are associated with the cytoskeleton it is possible that SRAPs can alter cell dynamics. The arrangement of the cytoskeleton has been shown to be altered in *Medicago trunculata* L. grown with *Glomus versiforme* (Karston) Bech (Blancaflor, 2001). Expression of two plant genes, a glycosylated cell wall protein and a xyloglucan endotransglycosylase, both of which indicate changes in wall architecture, was also increased in *Medicago trunculata* roots grown with *Glomus versiforme* (van Buuren, 1999). Other membrane proteins that show up-regulation in response to mycorrhizal association are plant and fungal H+ ATPases (Gianinazzi-Pearson et al., 2000). These membrane proteins are involved in transfer of phosphate and other nutrients.

The presence of H+ ATPase in both fungus and plant provides evidence that nutrient transfer between the two organisms is critical to successful symbiosis. Carbon is one such nutrient and its metabolism is dynamic in developing mycorrhiza. In the plant host, sucrose levels in the roots rapidly lower and are concomitant with an increase in fungal sugars. The depletion of sucrose is due to an increase in sucrose synthase and invertase activity in cell wall and cytoplasm of *Trifolium repens* L. root cells (Wright et al., 1998). One fungal sugar that accumulates in mycelium of mycorrhizal fungi is trehalose. In cultures of *Amanita muscaria* (L. ex Fr.) Hook grown in glucose, trehalose accumulates early and rapidly with a simultaneous decrease of glucose in the medium (Hoffmann et al., 1997). In field grown mycorrhiza of *Pinus resinosa* Ait., trehalose accumulates in winter months but is depleted during warmer months (Koide et al., 2000). In *Pisolithus tinctorius* (Pers.) Cok. and Couch trehalose concentration dropped in mycelium associated with roots of *Eucalyptus* (Martin et al., 1998).

The preceding discussion illustrates the highly specialized and complex relationship between orchids and their associated fungi. Inherent with this relationship are changes in gene expression in both the orchid and the fungus. These changes are evidenced in several ways including the peloton structure of the fungus after infection, a form which is only found when the fungus grows in the orchid. The fungus responds to the plant and changes its growth, a function that requires altered gene expression. The digestion of the fungus by the plant also suggests changes in gene expression as the

enzymes to digest the fungus are produced only in cells with fungus at a certain level of growth. Blakeman et al. (1976) showed that oxidative enzymes had peak activities in germinating seeds and that the activity of these enzymes coincided with maximum oxygen uptake and peleton digestion. Whether these enzymes are newly synthesized, which would indicate gene expression, or are made active by phosphorylation is not known. Further, to control infection and spread of the fungus, an array of plant genes must be expressed that ultimately limits fungal growth. As in other systems, there is probably no single gene response, but a cascade of genes activated by a signal that eventually leads to a response. Many questions can then be raised; What triggers activation of genes? How are these changes effected? What genes are intermediaries in the response? What biochemical pathways are affected by these genes?

Symbiotic seed germination has proven effective for seed germination of temperate terrestrials. Still more work needs to be done on the interaction of fungus and plant if a true understanding is to be obtained. It seems obvious that the fungus elicits changes within the plant and, since the orchid seed germinates prior to infection, plants must stimulate the fungus. At the heart of these issues are changes in the proteome of both fungus and plant which are mediated through gene expression. A more comprehensive knowledge of fungal plant interactions can be gained through analysis of these genes expressed by both plant and fungus during symbiotic germination. Peterson et al. (1997) suggested that the orchid-fungus relationship "may prove very useful in determining the molecular events that underlie the pronounced structural changes that occur in both symbionts during mycorrhiza formation."

Pathogenic and Symbiotic Considerations

Plants interact with a variety of organisms throughout their lives and these interactions can result in parasitism, symbiosis, or they may have no effect on the plant. Of the symbiotic interactions, the mycorrhizal interaction is by far the most widespread. Some have stated that the formation of this symbiosis allowed plants to colonize land (Selosse and Le Tacon, 1998). This would then suggest that symbiosis predates parasitism/pathogenesis, or that pathogenic interactions evolved from symbiotic ones. That symbiosis and pathogenesis share, at some level, commonalities is not a new concept. Indeed, as Ausubel and Bisseling (1999) point out, similarities can be seen during initial contact, establishment of infection and in the formation of new structures. The symbiotic interaction even initiates defense responses though these are curtailed at a later point. With this parallel between pathogenic and symbiotic pathways, it is possible that with an increased understanding of plant-fungal symbiosis we can better understand pathogenesis. It has been suggested that a model system would serve well to promote our understanding of plant symbiotic interactions. *Medicago trunculata* has been proposed as one such model in its symbiotic relationship with *Glomus* species. However, this form of endomycorrhizae, while widespread, is rather ancestral. Another model could be orchid though the relatively large genome size, difficulties in seed germination and relatively long life span preclude it from use as a general model.

Though there are problems associated with the use of orchids as a model for studies on mycorrhizal symbiosis, there are some aspects not found in other plants, which make orchids essential for such studies. First, all orchids are mycotrophic at some stage of their lives. That is, the orchid is completely dependent on the fungus for its carbon.

This would suggest that the orchid is capable of subverting fungal defense or of using symbiotic responses to its own advantage. Some orchids are myco-parasites, needing a fungal partner for the entire life of the plant. This display of reverse parasitism suggests again that orchids have the potential to utilize symbiotic interactions for their own purposes and are able to subvert fungal defenses. Second, some fungi associated with orchids are pathogens on other plants. This suggests the similarity of pathogenesis and symbiosis and that orchids can use fungal pathogenesis to establish symbiosis. The orchid is able to overcome the pathogenic response which makes them good candidates to look for novel resistance mechanisms. Third, the orchid mycorrhizal symbiosis is considered to be a more derived symbiosis and as such shares aspects in common with symbiosis in several other derived plant families (particularly myco-parasitic members of monocot families Triuraceae, Burmanniaceae, and the dicot family Gentianaceae). All the plants in this group form intracellular fungal structures that are similar to the orchid pelotons. These plants also exhibit varying degrees of myco-parasitism. That these adaptations have arisen several times in different plant families suggests that the mechanisms to predate fungi are universal. It may also mean that a plant's ability to overcome fungal defense or even subvert fungal virulence is latent in all plants. Interestingly, more primitive plants (mosses, ferns and liverworts) display a similar form of mycotrophy in the gametophytic stage. Even lichens are fully capable of subverting the fungal partner in what has been described as "a spectacular display of parasitism" (Ahmadjian, 1973). It is important to note, however, that orchids are not immune to fungal attack, but have evolved to curtail certain pathogens for their own use.

Molecular Biological Considerations

Differential display using the polymerase chain reaction (DD-PCR) was first described by Liang and Pardee (1992). It has since become the most widely used method for identifying genes expressed at some developmental stage or in response to some stimuli. The method as described by Liang and Pardee (1992) uses pairs of primers to randomly amplify mRNAs at any given time. Since the same primer pairs are used for separate cells grown under different conditions, the overall banding patterns should be similar; however, because growing conditions are different, some cDNA bands will be present or absent in one cell extract only. These bands represent genes activated or silenced by the treatment. Though the sensitivity of PCR does allow for false positives, screening methods are available to minimize them. Recently, amplified fragment length polymorphisms has been used for differential display analysis (AFLP-DDPCR). The greater stringency afforded by this method helps eliminate unnecessary bands.

The primers used in DD-PCR consist of an oligo dT and a 10 nucleotide upstream primer. The oligo dT primer has 2 additional bases at its 3' end which serve to anchor the primer to the 5' end of the mRNA's poly-A tract. The upstream primer is random, though some sequences function better than others. The fragment sizes generated by these primers range from 100 to 500 nucleotides and are easily separated on a sequencing gel by electrophoresis (Liang and Pardee, 1992). From one primer pair, between 50 and 100 bands can be generated (Liang and Pardee, 1992). Combinations of primer pairs will thus provide a suitable subset of the mRNAs expressed in the cell.

In AFLP-DDPCR, the cDNA is restricted with a frequent cutter and a rare cutter. Adapters are then ligated to the ends of the cDNA. Primers designed to these adapters

are then used in PCR. In theory, only fragments cut once by each enzyme should be amplified via PCR. The stringency produced by this method is believed to reduce false positives. It is also designed to produce fragments that are more likely to come from coding regions of genes.

Seed research is one area where DD-PCR is being used. Johnson et al. (1995) used DD-PCR to identify genes associated with dormancy in *Avena fatua* L. DD-PCR has also been used to find genes expressed in both the plant and fungus during symbiotic infection of *Pisum sativum* L. by *Glomus mosseae* (Nicol and Gerd.) Gerd. and Trappe (Martin-Laurent et al., 1997), in the fungus during symbiotic infection of *Hordeum vulgare* L. by *Glomus intraradices* Schenck and Smith (Delp et al., 2000), and during the pathogenic infection of *Lycopersicon esculentum* L. by *Botrytis cinerea* Pers.: Fr. (Benito et al., 1996). The study with *Botrytis cinerea* and *Lycopersicon esculentum* led to the identification of fungal genes activated during the infection process. Differential display has also been used successfully in orchids. Yu and Goh (2000) identified several genes including one belonging to the MADS box family associated with transition to flowering in a hybrid *Dendrobium*.

Orchid molecular biology is a young and burgeoning field (Kuenhnle, 1997). Molecular techniques have been used to deduce systematic relationships (Cameron et al., 1999; Cox et al., 1997; Neyland and Urbatsch, 1996; Whitten et al., 2000) and mechanisms of post-pollination ovule development in orchid (Nadeau et al., 1996; O'Niel et al., 1993). Some research is now being directed toward creating transgenic orchids that facilitate studies on molecular physiology. Many plant physiological studies use transgenic plants to uncover physiological processes. For example, Kakimoto (1996) used T-DNA (mediated by *Agrobacterium tumafaciens* (Smith and Townsend) Conn) to identify genes involved in cytokinin signaling. Also, antisense constructs allow researchers to knock-out the function of a particular gene and screen for altered phenotype. However, these studies cannot be undertaken without methods to introduce genes into plants. Nan and Kuehnle (1995) showed that several methods could be used for gene transfer into orchids with microparticle bombardment being the most effective. Knapp et al. (2000) transformed species of *Brassia*, *Cattleya* and *Doritaenopsis* using microprojectile bombardment and produced plantlets with stable expression of the *bar* gene. Further, Nan et al. (1997) found that *Dendrobium* orchids contained an elicitor of *Agrobacterium* virulence genes indicating the possibility of using *Agrobacterium* as a vector for gene transfer. Belarmino and Mii (2000) transformed a hybrid *Phalaenopsis* using *Agrobacterium* and showed that GUS activity could be detected in regenerated plantlets. As exciting as these advancements are, their use may be limited by poor seed germination preventing the formation of homozygous transgenics.

Rationale and Significance

The orchid mycorrhizal relationship is fairly unique in the plant kingdom. However, it shares aspects in common with other types of mycorrhiza. Some orchid mycorrhizal fungi are pathogens of other plants suggesting that the orchid is able to circumvent or utilize possible virulence pathways. Furthermore, all orchids are mycotrophic at some stage in their lives, which again suggests that the orchid is able to control the infection process. These two points make the orchid an excellent model to

study plant-fungal interactions. It is possible that orchids have evolved to utilize pathogen virulence as a means to establish symbiosis. By studying the orchid mycorrhizal process we can see how it is similar and how it differs from pathogenesis. It will then be possible to develop new strategies to overcome pathogenic interactions in other plant species. By understanding how orchids and fungi are able to form a partnership in which the orchid is the dominant partner, it will be possible to analyze the evolution of plant fungal interactions and gain greater insight into symbiotic and pathogenic interactions.

As mentioned, native species of orchids are an exciting new crop for the nursery and landscape industry. However, wild collection has decimated some species populations, thus causing them to be declared endangered species that are restricted by CITES treaty from collection or sale, unless grown from seed or meristem clones. New techniques for propagation and production must be implemented so that these plants can be brought to an expanding market. Symbiotic seed germination offers a potential means of propagation though current efficiency is low. With identification of genes involved in the symbiotic germination process, a greater understanding of seed germination can be had. The results of such research will allow for the formulation of new methods to germinate native, terrestrial orchids. The benefits of such a program are: to bring these plants to a demanding public and to preserve these species for future generations. Also, easier sexual production methods will allow the breeding potential of native orchids to be realized bringing superior garden plants to the nursery industry.