THE EFFECT OF CONDITIONALLY DISPENSABLE CHROMOSOMES OF *NECTRIA HAEMATOCOCCA* MPVI ON RHIZOSPHERE COLONIZATION AND THE IDENTIFICATION OF A GENE CLUSTER FOR HOMOSERINE UTILIZATION

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As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Gerard Joseph White entitled The effect of conditionally dispensable chromosomes of *Nectria haematococca* MPVI on rhizosphere colonization and the identification of a gene cluster for homoserine utilization and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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SIGNED Gerard Joseph White

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DEDICATION

All that is good I dedicate to Jorge Zamora.

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ABSTRACT

The habitat diversity of the fungus *Nectria haematococca* MPVI has been shown to be due in part to conditionally dispensable (CD) chromosomes that carry habitat-defining genes. From a biological perspective, the CD chromosomes are analogous to plasmids that possess genes that determine the habitats of plant-associated bacteria. This study establishes that the *N. haematococca* CD chromosome that contains the genes for Pea Pathogenicity (*PEP* cluster) also carries genes for the utilization of homoserine, an amino acid found in pea root exudates. Competition studies presented here demonstrate that an isolate that lacks the *PEP* cluster, but carries a portion of the CD chromosome containing the homoserine utilization (*HUT*) genes, is more competitive in the pea rhizosphere than an isolate without the CD chromosome.

Further competition studies show that both the *PDA1* and *PDA6* CD chromosomes confer a competitive advantage in the rhizosphere of soybean, whereas only the *PDA6* CD chromosome confers a competitive advantage in the rhizospheres of tomato and alfalfa, and only the *PDA1* CD chromosome confers a competitive advantage in the rhizosphere of pea. These studies suggest the presence of genes on the *PDA6* and *PDA1* CD chromosomes that enhance the ability of *N. haematococca* to expand its habitat and support the idea that fungal CD chromosomes are analogous to host-specifying plasmids in plant-associated bacteria.

Transformation, insertional mutagenesis, and bioinformatics were used to identify a cluster of five genes on the *PDA1* CD chromosome that was responsible for the HUT phenotype in *N. haematococca.* One of the genes was found only in *N. haematococca*,

another was a fungal transcription factor, and the other three had homologs involved in the synthesis of the amino acids methionine, threonine, and isoleucine, in which homoserine is an intermediate. Competition experiments that compared isolates with or without the *HUT* cluster showed that the *HUT* cluster is responsible for increased competitive ability of HUT^+ *N. haematococca* isolates in the rhizosphere of pea. This study establishes that homoserine utilization can be a rhizosphere competency trait for *N. haematococca* and, to our knowledge, is the first example of a rhizosphere competency trait identified in a fungus.

I. INTRODUCTION

The rhizosphere

The term “rhizosphere”, from the Greek word “rhiza”, meaning root, and “sphere”, meaning field of influence, was first introduced by Hiltner (1904), and defined as the zone of soil immediately adjacent to legume roots that supports high levels of bacterial activity. Since its first use, however, the term “rhizosphere” has been broadened and is now usually assumed to extend from the root surface, the “rhizoplane”, out into the soil for up to a few millimeters, or a few centimeters for some desert and sand dune plants (Campbell and Greaves 1990).

The “rhizosphere effect” is characterized as a phenomenon in which microbial growth and activity is stimulated due to the effect of plant roots (Foster 1986). What creates this rhizosphere effect? Plant roots have the ability to exude a complex and broad assortment of chemical compounds into the rhizosphere. Plant root exudates may include low molecular weight compounds such as amino acids, sugars, organic acids, nucleotides, plant hormones, vitamins, and phenolics, as well as high molecular weight compounds such as proteins and mucilage (reviewed in Lynch 1976 and Lynch 1990). Also found in the rhizosphere are gases such as CO_2 and ethylene, cell parts from autolysis of cells, root border cells, and eventually larger parts such as root hairs and roots themselves (Kang and Mills 2004).

Studies have shown that between 30-60% of net photosynthetic carbon is transferred to roots of various annual plant species; between 40-90% of that carbon is then lost as rhizodeposition in the soil and respiration from the roots and associated

microorganisms (reviewed in Lynch and Whipps 1991). Although we say that the carbon is "lost" in root exudates, it is more accurate to state that the carbon is an essential component of the communication between plants and rhizosphere organisms, and an essential nutrient for rhizosphere mircro-organisms. The colonization of roots by symbiotic root inhabitants such as *Rhizobium* spp. and mycorrhizal fungi is, in turn, beneficial to those plants with which they form partnerships. For example, infection of legume roots by *Rhizobium* spp. results in improved nitrogen nutrition for the plant via N_2 fixation (Lynch and Whipps 1991). The majority of plant species benefit from mycorrhizal fungi by improved mineral nutrition and drought tolerance (Lynch and Whipps 1991). In return, the *Rhizobium* spp. and mycorrhizal fungi obtain carbon nutrients from the plants. Minchin et al. (1981) found that during the life of a range of different grain legumes, between 30-50% of the net photosynthetic carbon enters the roots and 63-79% of that carbon is then lost via rhizodeposition and respiration. Based on the results of various studies (Martin 1977, Haller and Stolp 1985, Heulin et al. 1987, Whipps 1990), Farrar et al. (2003) estimated that approximately 40% of photosynthetically fixed carbon is transferred to the roots; 15%, 19%, and 35% of that carbon is then transferred to mycorrhizal fungi, lost via exudation, and lost via respiration, respectively (Figure I.1). Estimates of rhizodeposition vary greatly and the methods used to determine C loss into the rhizosphere are tedious and difficult (Newman 1985). It is commonly reported that approximately 20% of photosynthetically fixed carbon is transferred to the rhizosphere through root exudates (Whipps 1985).

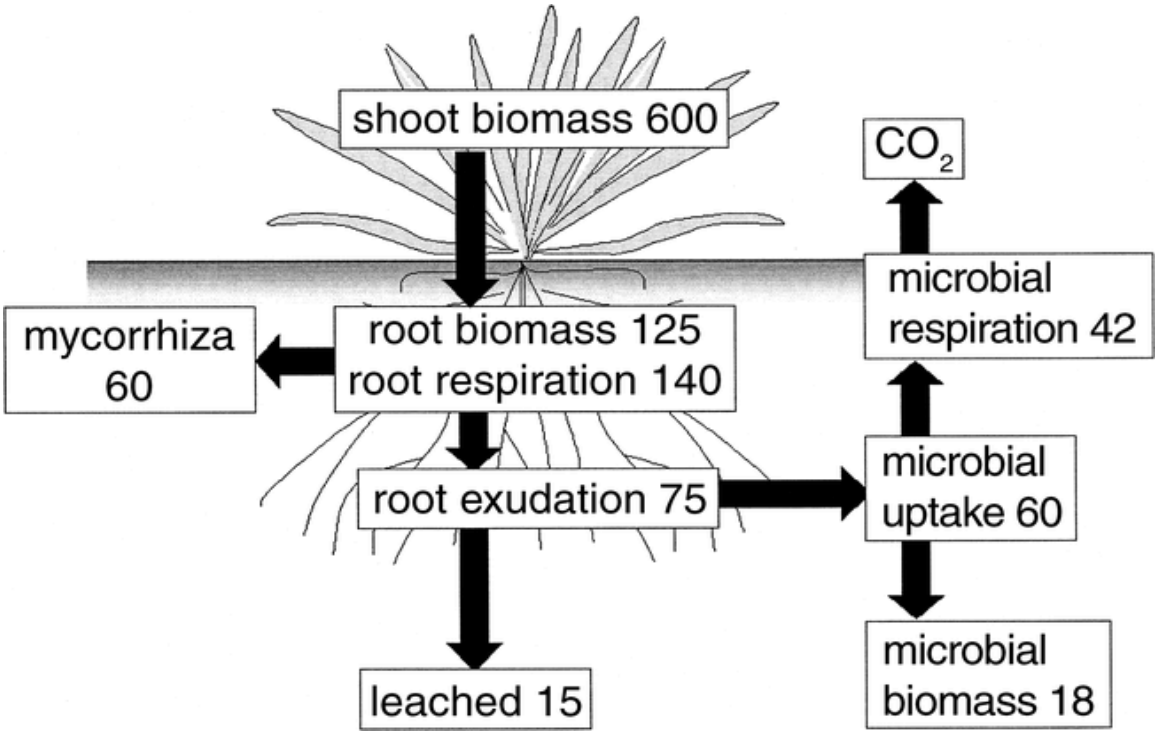

Figure I.1. The partitioning of C within the plant and the distribution of root exudates within the soil after the net fixation of 1000 C units. From Farrar et al. 2003. Used with permission.

Composition of root exudates

There are five main types of plant root exudates: diffusates, secretions, lysates, gases and mucilage (Grayston and Campbell 1996). Diffusates are low molecular weight, water-soluble compounds such as sugars, organic acids, and amino acids that passively diffuse from the root interior to the rhizosphere as a result of concentration gradients between the interior and exterior of the root (Benizri et al. 2001). Secretions are composed of low or high molecular weight compounds that are actively secreted by the roots in response to electrochemical gradients (Bowen and Rovira 1999, Benizri et al. 2001). Lysates consist of organic materials released into the soil after autolysis of dead cells (Benizri et al. 2001). Ethylene, carbon dioxide, and hydrogen cyanide are the main gases exuded from plant roots (Benizri et al. 2001). Plant mucilage, which is referred to as "mucigel" if it contains microbial mucilage, is composed of polysaccharides and polygalacturonic acids and helps roots penetrate into the soil (Benizri et al. 2001).

The composition of root exudates vary greatly according to plant species, physiological conditions such as plant age, general health, and nutrient status, abiotic conditions such as light, temperature, soil structure, soil aeration, and water content, and microbial activity at the root surface (Hale 1979, Campbell and Greaves 1990). As well, any type of stress on plant growth may induce increased plant root exudation (Curl 1982).

Rhizosphere organisms

Because of the rich supply of organic compounds, the rhizosphere, in comparison to the bulk soil, supports a wide variety and number of microorganisms, including commensals, mutualists, and pathogens. These large populations of microorganisms may

include up to 10^{10} - 10^{12} bacteria, fungi, protozoa, etc. per cm^{-3} soil (Foster and Rovira 1978, Malajczuk 1979), although it is believed that less than 1% of the microorganisms observed via microscopy can be cultured on common media under standard conditions (Torsvik and Øvreås 2002, Handelsman 2004). Roots exudates determine which organisms reside in the rhizosphere, i.e., the number and variety of organisms in the rhizosphere are related either directly or indirectly to root exudates and thus vary according to the same environmental conditions that influence exudation (Rovira 1965, Curl 1982, Cook and Baker 1983). Bolten et al. (1993) have suggested that root exudation evolved in plants as a way to stimulate an active rhizosphere microflora. This hypothesis is supported by (i) the fact that some rhizosphere microorganisms are selected for by certain host plant genotypes, and (ii) certain rhizobacteria have the capacity to improve plant health and, hence, capacity for reproduction (Nehl et al. 1996). Regarding this ability of root exudates to modulate rhizosphere properties, and thus affect plant growth and development, Hawes et al. (2003) stated: "Often overlooked is the fact that root exudates as they occur in the rhizosphere are not a generic hydrolyzate of soluble amino acids and sugars available to be consumed by any organism in the vicinity. Instead, the composition and biological activity of the material delivered by plants is dictated by the genotype of the plant (Atkinson et al. 1975, Neal et al. 1970, 1973). Conversely, whether or not a given component of root exudate is available to be used by a given microorganism is dictated by the genotype of the microorganism in question."

This dependency of rhizosphere microorganisms on root exudates is quite clear since the numbers of microorganisms generally decrease as the distance from the root

increases. To measure the effect of the rhizosphere on a population of microorganisms, the number of microorganisms in the rhizosphere (R) and the number of microorganisms in the bulk soil (S, soil not influenced by the root) are compared. The resulting R/S ratio gives an estimate of how strongly the rhizosphere affects a population of microorganisms (Rouatt et al. 1960). The R/S ratio is especially useful in determining the rhizosphere competence, the ability of an organism to colonize the rhizosphere, of various organism-plant combinations (Rouatt et al. 1960). Many bacteria have a high R/S ratio, indicating clear growth stimulation by compounds in the rhizosphere. Previous studies have shown an R/S ratio of 24.0 for bacteria in the rhizosphere and non-rhizosphere soil of both wheat (*Triticum aestivum*) and red clover (*Trifolium pretense*) plants (Rouatt et al. 1960, Rouatt and Katznelson 1961). This phenomenon is also clear for fungi. For example, Rouatt et al. (1960) showed an R/S ratio of 12.0 for fungi in the rhizosphere and non-rhizosphere soil of wheat plants.

(i) Decomposers: Rhizosphere microorganisms play a part in the decomposition and subsequent mineralization of organic matter in the rhizosphere, thus converting it into inorganic, plant-available forms (van der Heijden et al. 2008). It is estimated that approximately 96-98% of soil nitrogen is contained in insoluble organic materials such as proteins, nucleic acids, and chitin, which are broken down into soluble organic N by extracellular enzymes produced by soil microorganisms (Schimel & Bennett 2004). The soluble organic N is then absorbed by free-living soil microorganisms, mineralized by soil microorganisms and liberated as inorganic N, or taken up by plants in the form of amino acids (van der Heijden et al. 2008). The activity of rhizosphere decomposers also

yields other compounds, including phosphates, sulfates, carbon dioxide, and water from organic materials.

Generally, it is believed that bacteria and fungi differ in their ability to use organic compounds, with bacteria decomposing simple organic substrates such as sugars, organic acids, and amino acids (Kent and Triplett 2002, De Boer et al. 2005), while fungi decompose more complex organic compounds such as cell wall debris consisting of cellulose and lignin (De Boer et al. 2005). However, saprobic fungi that have ruderal characteristics such as rapid growth, prolific spore production, and the ability to use only relatively simple photosynthetically fixed carbon compounds, appear to be common rhizosphere inhabitants (Newsham et al. 1995, Orazova 1999). Indeed, studies using ^{13}C-labelled grasses indicated that fungi make significant contributions to the decomposition of root exudates (Butler et al. 2003, Treonis et al. 2004).

(ii) Plant growth-promoting rhizobacteria: Plant growth-promoting rhizobacteria (PGPR) are specific strains of bacteria in the rhizosphere that enhance seed germination and plant growth. Mechanisms of plant growth promotion by rhizobacteria may be direct, indirect, or a combination of both. Direct plant growth promotion involves the secretion of plant growth regulators, such as plant hormones, which elicit root metabolic activities or the supply of root-derived nutrients (Burdman et al. 2000). For example, studies have shown that strains of *Azospirillum*, *Azotobacter*, *Bacillus*, and *Pseudomonas* produce gibberellin-like compounds in culture that were important to plant growth responses (Hussain and Vancura 1970, Steenhoudt and Vanderleyden 2000). Observed plant growth stimulation by *Azospirillum* inoculation appears to involve the

production of plant growth regulatory substances. Indeed, three types of plant growth promoting substances, auxins, cytokinins and gibberellins, have been detected in the supernatant of *Azospirillum* cultures (Tien et al. 1979, Reynders and Vlassak 1979, Bottini et al. 1989). Production of plant hormones by *Azospirillum* has been shown to cause changes in root morphology, which in turn may be related to increased mineral uptake (Kapulnik et al. 1985, Okon and Kapulnik 1986, Jain and Patriquin 1985). Direct plant growth promotion may also result from improved nutrition as a result of increased phosphate solubilization (Kucey et al. 1989, Chabot et al. 1998) or increased mineralization processes by PGPR.

Indirect plant growth promotion occurs when PGPR reduce or prevent the effects of plant pathogens by various mechanisms including the production of antimicrobial compounds, or competition for iron, nutrients, or colonization sites (Whipps 2001). As discussed above, the rhizosphere contains a variety of filamentous fungi with the ability to rapidly decompose easily degradable organic compounds. The presence of these fungi probably exerts a selection pressure on bacteria to compete for these nutrients. Rhizosphere-inhabiting bacteria have evolved a multitude of antifungal strategies, including production of inhibitory compounds such as cyanide, antibiotics, lytic enzymes and volatiles, as well as nutrient-sequestering compounds such as iron-chelating siderophores (Ahl et al. 1986, Bakker et al. 1990, Thomashow et al 1990, Keel et al. 1990 and 1992, Dowling and O'Gara 1994, Duijff et al. 1994, Handelsman and Stabb 1996, Whipps 2001, Weller et al 2002, Wheatley 2002).

(iii) Mycorrhizal fungi: Another important group of plant rhizosphere symbionts that enhance plant productivity by accessing limited soil nutrients are mycorrhizal fungi. It has been hypothesized that the development of plant-fungal mutualism, i.e., mycorrhizae, preceded the development of roots and was crucial to the ability of plants to colonize the land (Brundrett 2002). Mycorrhizal fungi are widespread and form symbiotic associations with the roots of all gymnosperms, about 80% of angiosperms, and about 70% of pteridophytes (e.g. ferns, club mosses, and horsetails) (de Boer et al. 2005).

Mycorrhizae enhance nutrient solubilization and absorption in the rhizosphere and expand the volume of soil the root can explore. Mycorrhizal fungi can provide drought resistance to plants by several proposed mechanisms, including increased water uptake due to hyphal extraction of soil water (Hardie 1985). A range of limiting nutrients, including N, phosphorus, copper, iron, and zinc are supplied to plants by mycorrhizae in exchange for carbon (van der Heijden et al. 2008). Experiments have shown that mycorrhizae can contribute to up to 90% of plant phosphorus uptake, which is especially important for plant species with a high phosphorus requirement such as legumes, or under conditions when plant productivity is strongly affected by phosphorus availability such as in the tropics (Jakobsen et al. 1992; Van der Heijden et al. 1998, 2006). Mycorrhizae have also been shown to increase plant uptake of metal ions present in very low concentrations, such as zinc (Faber et al. 1990, Pacovsky 1986), copper (Gildon and Tinker 1983, Manjunath and Habte 1988), and cobalt (Killham 1985, Rogers and Williams 1986).

Numerous studies concerning pathogen-mycorrhizal interactions have focused on developing mycorrhizal fungi for biological disease control (reviewed by Harrier and Watson 2004, Whipps 2004). Whipps (2004) identified five major modes of action: (1) mycorrhizae may directly compete with the pathogens for photosynthates in the rhizosphere or for space on the roots; (2) mycorrhizae may produce low levels of antibiotics, phenolics, or other defense molecules that inhibit the pathogens; (3) mycorrhizae may enhance or alter plant growth, nutrition, and morphology as a result of increased nutrient uptake, drought tolerance, and decreased toxicity to salt and heavy metals; (4) colonization of the roots by mycorrhizae may enable the plant to respond more rapidly to subsequent pathogen attack by resistance mechanisms pre-activated by the mycorrhizae (induced resistance and systemic acquired resistance); and (5) mycorrhizae may stimulate the development of soil microbiota antagonistic to pathogens, as shown by dual inoculations of *Glomus* spp. with a variety of bacteria including *Azospirillum* spp., *Bacillus* spp., *Pseudomonas* spp. and *Rhizobium* spp., and fungi, such as *Gliocladium* spp. and *Trichoderma* spp., which have resulted in either improved plant growth or decreased severity of several pathogens (reviewed by Bending et al. 2006).

(iv) Nitrogen-fixing bacteria: Nitrogen-fixing bacteria in the plant rhizosphere are important regulators of plant productivity because plants cannot fix atmospheric N_2 which is, together with phosphorus and potassium, one of the main elements limiting plant productivity (Chapin 1980). Rhizobia (species of *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, and *Sinorhizobium*) form nodules on the

roots of plants, take N_2 from the air, and reduce it to ammonia, which is a form usable by the plant, while the plant provides nutrients and a competition-free home for the bacteria. The rhizobial-legume symbiosis is generally very specialized, with both the plant and rhizobium having narrow host ranges. Exceptions include *Rhizobium* sp. NGR234, which nodulates over 110 genera of legumes (Pueppke & Broughton, 1999) and *Phaseolus vulgaris* (the common bean), which is nodulated by at least twenty species of rhizobia (Michiels et al. 1998). Formation of the symbiosis is dependent on an elaborate signal exchange with only the correct combination giving rise to efficient symbiosis (Fauvart and Michiels 2008). Rhizobia in the rhizosphere are chemotactically attracted to host plants upon detection of plant root exudates such as flavonoids, phenolics, sugars, dicarboxylic acids and amino acids, and subsequently adhere to and colonize the root surface (Brencic and Winans, 2005). Flavonoids in plant root exudate also induce the expression of a series of rhizobial nodulation (*nod*) genes, resulting in the synthesis and release of Nod factors. Nod factors induce root hair curling, division of root cortex cells, and localized degradation of root hair cell walls, all steps leading to the entry of rhizobia into the root hairs and subsequent development of root nodules. Once nodules have developed, the rhizobia differentiate into bacteroids and are able to fix nitrogen in the environment provided by the nodule interior.

(v) Pathogens: Soil-borne plant pathogens are micro-organisms that cause diseases to the roots, other underground parts, and vascular systems of plants. It is important to remember that beneficial interactions of roots with soil micro-organisms such as N_2-fixing bacterial and mycorrhizal fungi are much more frequent than

interactions between roots and pathogenic micro-organisms (Katan 1996). However, although plant pathogenic agents constitute only a small fraction of the total population of soil micro-organisms, their impact on agricultural plants can be enormous, leading to complete destruction of plants and loss of yield (Katan 1996).

Soil-borne plant pathogens include fungi, bacteria, and nematodes. For the purpose of this study I will discuss only the fungal and Oomycete soil-borne plant pathogens. These cause three major types of diseases: (1) Damping-off disease of seedlings that develop at the pre- or post-emergence stages of germination (e.g. *Fusarium* spp., *Rhizoctonia* spp., *Pythium* spp., and *Phytophthora* spp.); (2) Root, crown, and foot rots (e.g. *Fusarium* spp., *Phoma* spp., *Thielaviopsis* spp., *Phymatotrichum* spp., *Cochliobolus* spp., *Gibberella*, *Gaemannomyces* spp.; and (3) Vascular wilt diseases in which the pathogen penetrates the roots and moves upwards through xylem vessels, plugging these vessels and disrupting movement of water through the plant (e.g., *Fusarium* spp. and *Verticillium* spp.).

When Hiltner (1904) first talked about the rhizosphere he stated, "The nutrition of plants in general certainly depends upon the composition of the soil flora in the rhizosphere…If plants have the tendency to attract useful bacteria by their root excretions, it would not be surprising if they would also attract uninvited guests which, like the useful organisms, adapt to specific root excretions." Fungistasis, the property of natural soils to inhibit germination of fungal propagules (Dobbs and Hinson 1953), is overcome by pathogen propagules in the plant rhizosphere (Katan 1996). Root exudates may affect pathogen propagules by inducing their germination in the presence of the host,

by attracting motile propagules (e.g. zoospores) to the roots, by stimulating pathogen growth, and by formation of infection structures (Katan 1996). In general, root exudates are non-specific regarding which species' propagules they stimulate to germinate (Coley-Smith and King 1969). There are exceptions, however. Previous studies showed that volatile thiols and sulfides from soil microbial degradation of sulfoxides present in *Allium* root exudates trigger the germination of *Sclerotium cepivorum* (a pathogen of *Allium* species) sclerotia (Coley-Smith and King 1969, Cooley-Smith and Cooke 1971). Similarly, Nelson (1987) showed that volatile seed exudates stimulate the germination of *Pythium* spp. sporangia. Nicol et al. (2003) reported that ginsenosides, triterpene saponins found in American ginseng root exudates, enhance the growth of two ginseng pathogens, *Phytophthora cactorum* and *Pythium irregulare*. Of particular interest to the current study, Ruan et al. (1995) found that various specific host flavonoids and isoflavonoids of pea and bean stimulated *Fusarium solani* f. sp. *pisi* (host = pea) and f. sp. *phaseoli* (host = bean) macroconidia germination. Their results indicated that the germination response was highly specific for certain host plant flavonoids, not merely due to utilization of these flavonoids as nutritional sources (Ruan et al. 1995). This is an example of how a plant pathogen has adapted to recognize host plant chemicals, a necessary component of the response of a highly specialized pathogen to its host.

The fungus *Nectria haematococca*

Nectria haematococca Berk. & Br. (anamorph: *Fusarium solani*) is a soil-borne, plant pathogenic, filamentous fungus in the phylum Ascomycota of the class Pyrenomycetes (Booth 1971). *Nectria haematococca* encompasses a genetically diverse

group of fungi including both homothallic (self-fertile; i.e. fertile perithecia can develop from the pairing of isolates of the same mating type) and heterothallic (self-sterile; i.e. fertile perithecia develop only when two isolates of opposite mating types are paired) forms. The heterothallic isolates require opposite alleles at the mating type (*MAT*) locus for a fertile cross and are subdivided into seven incompatible groups called mating populations (MPI - MPVII) (Matuo and Snyder 1973). The current research deals with isolates of MPVI, which have been assigned to this MP by their ability to cross with reference strains (VanEtten 1978).

Members of MPVI are found in diverse biological habitats and exist as soil-inhabiting saprobes, as saprobes in the rhizosphere of plants, or as pathogens that cause disease on at least one animal species (penaeid shrimp) and nine plant species including both monocots (i.e. maize, barley) and dicots (i.e. garden pea, chickpea, red clover, alfalfa, cottonwood, ginseng, mulberry, sainfoin, and tuliptree) (VanEtten and Kistler 1988). Previous studies have shown that the ability to cause disease on one host can be independent of the ability to cause disease on a different host, thus suggesting that different genes control host-specificity in *N. haematococca* MPVI (Matuo and Snyder 1972, VanEtten and Kistler 1988).

Cultural characteristics and spore production of *N. haematococca*

Macroscopic morphology of *N. haematococca* may vary significantly on different media. On potato dextrose agar colonies are usually fast-growing and are cottony with cream to white aerial mycelium. Sporodochia (slimy clusters of conidiogenous cells and macroconidia visible as raised areas on the medium) may form and are usually moist and

cream-colored but may be blue-green (Nelson et al. 1983). Hyphae are septate and hyaline. Conidiophores are simple (non-branched) or branched monophialides (phialides with a single opening) (Nelson et al. 1983). The optimal temperature for growth on agar media is approximately 30^{o}C, although disease will develop on pea and other host plant spp. at 18^{o}C and above, with an optimal temperature of 25-30^{o}C (Kraft and Roberts, 1969).

The two major asexual spore forms of *N. haematococca* are microconidia and macroconidia. *N. haematococca* microconidia, which are usually unicellular and uninucleate, are 2-5 by 8-16 µm, hyaline, cylindrical to oval, and generally formed in abundance in liquid media (Booth 1977). Multicellular macroconidia normally predominate on rich media such as that containing V8 juice. Macroconidia are 4.4-5 µm by 27-40 µm, hyaline, and curved, and have 3-5 septa (Booth 1977). All nuclei of a macroconidium are mitotic descendants of the same progenitor nucleus and are therefore genetically identical (Dickinson 1932). A third type of asexual spore, the chlamydospore, is sometimes present. Chlamydospores of *N. haematococca* are hyaline, globose, smooth- to rough-walled spores that result from structural modification of vegetative hyphal segments or conidial cells (Booth 1977, Schippers and van Eck 1981). Survival in soil often depends on chlamydospores, which have the ability to withstand adverse environmental conditions (Nash et al. 1961). Germination of over-wintering chlamydospores is stimulated by nutrients released from germinating pea seeds (University of Illinois Extension Report on Plant Disease No. 911).

Ascospores, the sexual spores of *N. haematococca*, form inside dark red, spherical perithecia (ascocarps). Perithecia of *N. haematococca* MPVI in the field have been reported only on the branches of mulberry trees in Japan (Matuo and Snyder 1972).

Fusarium root rot distribution, symptoms, and control

Fusarium root rot, caused by *Fusarium solani* f. sp. *pisi,* is a world-wide disease of considerable economic significance. Fusarium root rot can seriously reduce the yield and quality of the crop (Kraft and Roberts 1969). It was first reported as a serious disease of pea plants in Minnesota in 1918 (Bisby 1918), in Wisconsin in 1923 (Jones 1923), and in Europe at around the same time (Buxton 1955).

Fusarium root rot is favored by warm temperatures and acidic, poorly fertilized soils (Bost 2006). The disease tends to be evenly distributed through large portions of a field (Bost 2006). In pea seedlings the initial center of infection by this pathogen is the area where the cotyledons are attached, the below-ground epicotyl, and the upper taproot (Bywater 1959). Infection extends upward to the soil line and downwards to the roots. Seedlings often die within a week of emergence but if the plant does not die immediately, rootlets may develop above the dead area, which may allow the plant to survive (Bost 2006). Reddish brown lesions forming in the primary and secondary roots may turn dark brown, coalesce, and develop lengthwise cracks (Univ. IL Ext. Rep.). As the infected root usually remains firm, this disease is often called “dry root rot” (Bost 2006). Above-ground symptoms of this disease include yellowing of basal foliage, wilting, stunted growth, and the presence of few, poorly filled pods (Bost 2006, Univ. IL Ext. Rep.).

There is no completely satisfactory control for Fusarium root rot once the fungus is introduced and becomes prevalent in the soil. Cultural controls include planting into warm, firm seedbeds to promote rapid emergence, use of healthy seed with high germination capacity, avoiding plant stress (e.g., avoiding over- and under-watering, crowding, deep planting, soil compaction, and mechanical damage to seeds, roots, and stems, and ensuring high soil fertility), and following a crop rotation scheme (Univ. IL Ext. Rep.). Nematodes can increase the severity of Fusarium root rot and should be controlled by crop rotation or other means (Bost 2006). Chemical controls involve treatment of seeds with protectant fungicides such as metalaxyl, captan, thiram, or a combination of fludioxonil and metalaxyl to protect the seedlings in the early stages of plant establishment (Agric. Agri-Food Canada 2005). No commercial cultivars of peas are currently available that are highly resistant to Fusarium root rot, although some have low/moderate resistance (Agric. Agri-Food Canada 2005).

Conditionally Dispensable (CD) chromosomes of *N. haematococca*

"Conditionally dispensable" (CD) chromosomes are supernumerary chromosomes that can be lost without affecting axenic growth (Miao et al. 1991). *N. haematococca* CD chromosomes of interest in the current study were identified by the loss of particular genetic traits combined with chromosome loss as determined by pulsed field gel electrophoresis (Miao et al. 1991, Covert 1998). CD chromosomes of *N. haematococca* MPVI differ from the more classically known supernumerary chromosomes, "B" chromosomes (Camacho et al. 2000, Jones et al. 2008), in that they have been shown to carry functional genes such as genes for pathogenicity, antibiotic resistance, and the

utilization of unique carbon and nitrogen sources (VanEtten et al. 2001). CD chromosomes have been identified in several fungal species including *Nectria haematococca* (Miao et al. 1991a, Kistler and VanEtten 1984, Miao et al. 1991b), *Cochliobolus heterostrophus* (Tzeng et al. 1992), *Colletotrichum gloeosporioides* (Masel et al. 1993, Masel et al. 1996), *Gibberella fujikuroi* (Xu et al. 1995), *Cochliobolus carbonum* (Ahn and Walton 1996), *Magnaporthe grisea* (Orbach et al. 1996), and *Alternaria alternata* (Johnson et al. 2001). Although the origin of CD chromosomes in fungi is unknown, they may result from horizontal transfer from genetically distinct sources (Masel et al. 1993).

The first CD chromosome to be described in a fungus was the 1.6Mb *PDA6-1* chromosome of *N. haematococca* MPVI, which could be lost during meiosis without apparent effects on axenic growth (Miao et al. 1991). Since then, studies have shown that the *PDA6-1* CD chromosome also carries a gene (*MAK1*) for detoxifying the chickpea phytoalexins maackiain and medicarpin (Covert et al. 1996) and that loss of the *PDA6-1* CD chromosome or disruption of the *MAK1* gene equally reduces the virulence of *N. haematococca* on chickpea (Enkerli et al. 1998). Another *N. haematococca* gene, *PDA1*, is required for virulence on pea and is located on a different 1.6Mb CD chromosome than *MAK1* and *PDA6-1* (Funnell and VanEtten 2002, Wasmann and VanEtten 1996). While disruption of *PDA1* reduced virulence on pea, loss of the entire *PDA1* CD chromosome made the isolate almost completely non-pathogenic on pea (Wasmann and VanEtten 1996), thus suggesting that the *PDA1* CD chromosome contains additional pea pathogenicity (PEP) genes (VanEtten et al., 1994; Wasmann and

VanEtten, 1996). Further studies have shown that there is a cluster of pea pathogenicity genes (*PEP* genes) clustered around the *PDA1* gene and when the *PEP* cluster is deleted from this CD chromosome, the isolate is no longer pathogenic on pea (Han et al. 2001, Wasmann and VanEtten, 1996).

The *PDA1*-CD chromosome also has a gene(s) (*HUT*), located outside the *PEP* cluster, that allows the utilization of homoserine, an amino acid found in large quantities in the root exudates of pea plants (Rodriguez et al. 2008, Appendix A). My hypothesis is that the *HUT* gene(s) confer a competitive advantage during colonization of the pea rhizosphere prior to the onset of the pathogenic interaction. This rhizosphere-competitiveness gene(s) increases the likelihood that the fungus will have colonized the pea rhizosphere at the right time to initiate disease. Although *N. haematococca* isolate 77-13-4, which possesses the gene(s) for homoserine utilization on its *PDA1*-CD chromosome, has been should to be able to utilize homoserine as a sole carbon and nitrogen source, it has not been shown that this characteristic gives that isolate a competitive advantage in the rhizosphere of pea. In this study I used a real-time PCR technique to enumerate *N. haematococca* isolates with and without the *PDA1* and *PDA6-1* CD chromosomes to determine if these chromosomes give the isolates possessing them a competitive advantage in the rhizospheres of pea and other plant species.

N. haematococca interactions with pea root border cells

Root border cells are viable, metabolically active cells that separate from the periphery of root caps as the roots grow through the soil (Hawes and Lin 1990). They are

experimentally defined as those cells that separate from the root upon brief contact with water and gentle agitation (Hawes and Wheeler 1982). The numbers of border cells from aseptically grown seedlings varies considerably, with fewer than ten on tobacco root tips and more than 10,000 on cotton root tips (Hawes 1990). Pea root tips were observed to have ~3500-4500 border cells (Hawes et al. 2003). Once released from the root cap, border cells are developmentally and morphologically distinct from the root cap and form a distinct physical and biological boundary between the root and the soil (Hawes and Lin 1990). Border cells remain alive at a considerable distance from the root cap (Vermeer and McCulley 1982) and are capable of independent behavior, as demonstrated by their ability to attract bacteria and fungi in host-specific manners (Hawes et al. 1998). Border cells, along with secretions from root cap cells, are major contributors of root exudates (Bacic et al. 1986, Chaboud 1983, Griffin et al. 1976, Miki et al. 1980, Morre et al. 1967). Various studies have shown that root border cells not only deliver much of the bulk weight of root exudates but they also produce and secrete a wide range of specific extracellular signals that can attract, repel, and control growth and gene expression in soilborne organisms (as reviewed in Hawes et al. 2003). It has also been shown that when root cap cells differentiate into border cells there is a change in their gene expression such that their protein profile becomes distinct from that of root cap cells (Brigham et al. 1995).

Gunawardena and Hawes (2002) showed that inoculation of young pea roots with *N. haematococca* spores resulted in infection of more than 90% of the roots, but the infection was confined to the region of elongation, with no evidence of lesion formation

in older tissues or root tips. They hypothesized that the region of elongation was the preferential site of infection because it is the first available site of newly-generated tissue that is not covered with a population of border cells whose function is to protect the root tip from infection (Hawes et al. 1998, 2000, Gunawardena and Hawes 2002). The *N. haematococca* hyphae were observed to grow quickly throughout the pea border cells, forming a sheath that detached as the root elongated, thereby leaving the tip uninfected (Gunawardena and Hawes 2002). Even microscopic lesions from peripheral root cap cells were seen to detach along with the detaching mantle (Hawes et al. 1998). Despite the close proximity of root tip and border cells, the tip often remained free of infection for days, whereas the border cells rapidly became infected by *N. haematococca* (Gunawardena and Hawes 2002). It has also been observed that colonization of pea root border cells by *N. haematococca* did not induce defense gene expression in the root tips, thereby emphasizing the physiological independence of the root cap from the border cells (Gunawardena and Hawes 2002).

Traditional enumeration techniques for soil/rhizosphere fungi

Various methods have been developed to detect and quantify fungi in plant and soil environments. One major problem with assessing individual species (or strains) of fungi in soil is that the soil environment consists of a very heterogeneous and usually complex microbial community, so that evaluating growth of a specific organism within a mixture of other fungi and bacteria is extremely difficult (Orr and Knudsen 2004). Methods of quantification based on serial dilutions and plate counts have traditionally been used to quantify fungi in soil (Parkinson et al. 1971), although it is well known that

numbers obtained from plate counts may not accurately reflect growth and spread of fungi in soil, and may not strongly correlate with fungal biomass (Garrett 1981, Lumsden et al. 1990, Dandurand and Knudsen 2002). Dilution plating techniques do not differentiate between different viable fungal propagules (e.g., microconidia, macroconidia, and hyphal fragments) that may generate colonies on selective media (Orr and Knudsen 2004). Because colonies may be generated from either single-celled propagules such as conidia, or from multi-celled hyphal fragments, it is extremely difficult to accurately quantify actual fungal biomass based on plate counts. Techniques have also been developed that are based on the quantification of specific biochemical components of fungal cells, such as phospholipid fatty acids (Federle 1986, Frostegård and Bååth 1996, Knudsen 2002), ergosterol (Seitz et al. 1979, Grant and West 1986, Bermingham et al 1995, Stahl and Parkin 1996, Montgomery et al. 2000), and chitin (Pacovsky and Bethlenfalvay 1982, Plassard et al. 1982). One issue with quantifying fungal cell components (e.g., chitin) to represent fungal biomass is that there may be false-positive results due to the presence of substances derived from either living or non-living non-fungal biomass (e.g., invertebrate exoskeletons contain chitin) (Grant and West 1986). Aside from the lack of specificity, quantities of fungal cell components do not necessarily correlate with fungal biomass due to various factors including variations in concentration with species, physiological state and age, and fungal sporulation and interference from enzymatic activities in plants (Sharma et al. 1977). Fungal biomass has also been determined using immunological methods (Dewey et al. 1997, Harrison et al. 1990, Karpovich-Tate et al. 1998, Newton and Reglinski 1993, Thornton et al. 1994),

although it is often difficult to obtain antibodies with the required specificity for particular fungal species (Ward et al. 1998). An additional problem with immunoassays is that they may be prone to false-positive reactions (Takenaka 1995, Srivastava and Arora 1997).

This study: Real-time PCR

In recent years, the polymerase chain reaction (PCR) method has emerged as a major tool to detect and identify fungal species via the detection of DNA and RNA. With PCR, oligonucleotide primers that are specific for the DNA of interest can be designed to distinguish closely-related fungal species, thereby offering the advantage of higher specificity than many other methods (Henson and French 1993). One disadvantage of conventional PCR is that it requires labor intensive and time-consuming post-amplification steps, such as running electrophoresis gels and image processing, which can be sources of inaccuracy and carry-over (Schena et al. 2004). Other disadvantages are that reaction components may become limiting before the end of the amplification and it is unreliable for quantitative analysis (Ginzinger 2002).

Higuchi et al. (1992, 1993) designed a system that detects PCR products as they accumulate. Their "real-time" system included the intercalating agent ethidium bromide in each amplification reaction, a thermal cycler modified to irradiate the samples with ultraviolet light, and a camera to detect the resulting fluorescence (Higuchi et al. 1992, 1993). During real-time amplification, increasing amounts of double-stranded DNA are produced, which subsequently binds the ethidium bromide, resulting in an increase in fluorescence. Plotting the increase in fluorescence versus the cycle number, provides

amplification plots that give a more complete picture of the PCR process than assaying product accumulation after a fixed number of cycles.

The main disadvantage of detection based on ethidium bromide (or other DNA-binding dyes) is that both specific and nonspecific PCR products generate a signal, thereby necessitating the use of melting curve analysis. Real-time PCR systems were subsequently improved by target-specific oligonucleotide probe-based, rather than intercalator-based, PCR product detection. These probes (e.g. Taqman®) are labeled with a donor fluorophore at the 5' end and an acceptor dye (quencher) at the 3' end. The fluorophore molecule absorbs light energy and is elevated to an excited state. In the absence of a quencher the fluorophore falls back to the ground energy state and releases the excess energy as fluorescent light. The quencher molecule accepts energy from the fluorophore and dissipates the energy (Didenko 2001). Holland et al. (1991) demonstrated that the 5'-nuclease activity of *Taq* DNA polymerase could provide a real-time method for detecting only specific amplification products. During real-time PCR the probe binds to its target sequence and is subsequently cleaved by the 5'-nuclease activity of *Taq* DNA polymerase when the enzyme extends from an upstream primer into the region of the probe. Cleavage of the probe releases both fluorophore and quencher into the reaction mixture, thereby resulting in an increase of fluorescence proportional to the amount of PCR product (Holland et al. 1991). Dependence on binding of the probe to its specific target DNA ensures that cleavage of the probe occurs only if the target sequence is being amplified (Schena et al. 2004). Further advantages of fluorescent probes over DNA-binding dyes are that the probes can be labeled with different distinguishable

reporter dyes for amplification and detection of more than one DNA species, and that they replace the potentially mutagenic ethidium bromide.

Real-time PCR, as it is currently used, combines thermal cycling with detection of the cleaved target-specific fluorescent probe and records a specific cycle number, represented by the "threshold cycle" (Ct), at which a statistically significant increase in the fluorescence (just greater than the background) can first be detected (Qi and Yang 2002). Because the Ct is inversely proportional to the log of the initial DNA template, the more template that is initially present in PCR samples, the smaller the Ct it takes to get to a point where the fluorescent signal is detectable above background. Quantitative data based on the Ct is more accurate than data derived from endpoint determinations because the Ct measurement is taken during the exponential phase of PCR amplification when efficiency has not yet been influenced by limiting reagents, small differences in reaction components, or cycling conditions (Qi and Yang 2002).

The power of real-time PCR is reflected in the use of this technique in various areas of scientific research. Real-time PCR has become an essential tool for the diagnosis and treatment of many human diseases (as reviewed in Kaltenboeck and Wang 2005, Wittek et al. 2007, Valasek and Repa 2005, Morshed et al. 2007, Millar et al. 2007, Ratcliff et al. 2007, Espy et al. 2006). It has also been used for detection and quantification of viruses, bacteria, fungi, and other microorganisms in the fields of food microbiology (as reviewed in Rodriguez-Lazaro et al. 2007, Gasanov et al. 2005, Deisingh and Badrie 2005, Hanna et al. 2005, Levin 2004) and environmental science (as reviewed in Zhang and Fang 2006, Straub and Chandler 2003, Gilbride et al. 2006,

Lebuhn et al. 2004, Anderson and Cairney 2004). It has been increasingly used to detect and/or or quantify viral (Mumford et al. 2000, Roberts et al. 2000, Lopez et al. 2003), bacterial (Schaad et al. 1999, Weller et al. 2000, Lopez et al. 2003, Salm and Geider 2004), and fungal (Bates and Taylor 2001, Bates et al. 2001, Böhm et al. 1999, Frederick et al. 2000, McCartney et al. 2003, Gao et al. 2004, Lievens et al. 2006, Luo et al. 2007, Cullen et al 2007) plant pathogens.

In the current study, real-time PCR was used to determine the quantities of *N. haematococca* isolates in the rhizospheres of various plant species. We anticipated that the use of real-time PCR would overcome the problems of traditional methods for quantifying fungal biomass in soil/rhizosphere.

Plant model systems used in this study: pea, soybean, diploid alfalfa, and tomato

Pisum sativum, the garden pea, is a flowering plant that belongs to the family Leguminosae (Fabaceae). *P. sativum* is a commercially important food crop and the seeds of the garden pea are widely consumed by both humans and livestock. Peas contain about 21-25% protein, high levels of carbohydrates, and high levels of the amino acids lysine and tryptophan (McKay et al. 2003). Pea plants may also be grown as a green manure to improve soil nitrogen levels or as a green fallow crop to protect the soil from erosion and improve soil quality (McKay et al. 2003). As well as being important as a valuable agricultural plant, *P. sativum* also serves as a model plant for genetic and physiological studies (Rick 1971). These include studies in the field of mutualistic plant-microbial interactions since *P. sativum*, like other legumes, forms mutualistic symbioses with both nitrogen-fixing bacteria and with beneficial arbuscular mycorrhizal fungi. In the case of

P. sativum, these symbioses are with the nitrogen-fixing bacterium *Rhizobium leguminosarum* bv. *viciae* and arbuscular mycorrhizal fungi belonging to the phylum *Glomeromycota*.

Soybean, *Glycine max*, belongs to the family Leguminosae and is the most important leguminous crop in the world due to its high content of high-quality protein for humans and livestock, as well as its oil that is used to produce food and industrial materials (Harada and Xia 2004). The United States is the world's leading soybean producer and exporter. US soybean production was valued at $18.0 billion in 2003/04, the second-highest value among American-produced crops (USDA Statistics). Soybeans accounted for approximately 90% of American total oilseed production, while other oilseeds such as cottonseed, sunflower seed, canola, and peanuts made up the remainder (USDA Statistics). Physiologically functional constituents in soybean seeds contribute significantly to human health, and because of its symbiotic relationships with nitrogen-fixing bacteria, soybean supplies nitrogen to soil (Harada and Xia 2004).

Medicago truncatula, diploid alfalfa, belongs to the family Leguminosae and is a close relative of *Medicago sativa* (tetraploid alfalfa) that is commonly grown as a forage crop in Australia (Center for Medicago Genomics Research, Samuel Roberts Noble Foundation Website). *M. truncatula* has been chosen as a model legume due to features such as its small, diploid genome (1.8×10^9 bp for the Jemalong cultivar; $2n = 16$), self-fertility, prolific seed production, rapid generation time (~3 months), and high transformation efficiency (Cook 1999, Barker et al 1990). The genotype Jemalong A17 is of special interest because the genome of this line is currently being sequenced (Zhou et

al 2004). Studies on syntenic relationships (comparisons of genome content and organization between organisms) have shown a high level of conservation between *M. truncatula*, *M. sativa*, and pea, and a moderate level of conservation between *M. truncatula*, soybean, and other legumes, thus providing the potential to transfer genomic information into crop improvement (Abranches et al. 2005). Disease development in *M. truncatula* challenged with various pathogens has been shown to be similar to that observed in other legume species, indicating that *M. truncatula* can be used as a model plant to study the molecular background of pathogenic interactions in these species (Nyamsuren et al. 2003).

Tomato, *Solanum lycopersicum* (formerly *Lycopersicon esculentum* Mill. var. *esculentum*), belongs to the Solanaceae family. It is an annual vegetable crop that is grown worldwide from the tropics to the Arctic Circle (Hancock 1992). In the United States, tomato ranks only behind potatoes in popularity among vegetables (Hancock 1992). US fresh market tomatoes were valued at $1.342 billion in 2004, while processed tomatoes were values at $719 million (USDA Statistics 2005). Most tomatoes grown today are hybrid varieties that have been bred for certain plant characteristics such as stress, insect, and disease resistances, and fruit characteristics such as uniform size and coloring, extended shelf life, and firmness (Atherton and Rudich 1986). Tomato, like corn, was an early model system for genetics and cytogenetics studies in plants (for review see Rick 1971). The Solanaceae family is unique among plants in that there have been no large-scale duplication events, such as polyploidy, early in the radiation of this family (SOL 2003). Polyploidy events, for example those resulting in tetraploid potatoes

and tetraploid tobacco, are all recent events and diploid forms of both of these species are still in existence (SOL 2003). As a result, microsynteny conservation among the genomes of tomato, potato, pepper and eggplant is very high (SOL 2003). The International Solanaceae Genome Project (SOL) is mapping the genome of tomato in order to provide a reference for interpreting the sequence organization of other Solanaceous crops as the basis of understanding how plants diversify and adapt to new environments (SOL 2003). Tomato was selected by SOL as a reference plant for several reasons: (1) it has the smallest diploid genome (950 Mb) for which homozygous inbred varieties are available; (2) there is an advanced BAC-based physical map of tomato available to start the sequencing; and (3) tomato is the most intensively studied Solanaceous genome due to its simple diploid genetics, short generation time, and routine transformation technology (SOL 2003).

Dissertation format

The research in this dissertation has been published (Appendix A), or will shortly be submitted for publication (appendix B and C). Because of this, according to the University of Arizona manual for dissertations format what would usually be chapters in a dissertation are put as appendices. The following section describes my research contribution to each of the appendices. The appendices' formats are according to the requirements of the journal to which they will be submitted.

II. PRESENT STUDY

APPENDIX A: THE SUPERNUMERARY CHROMOSOME OF *NECTRIA HAEMATOCOCCA* THAT CARRIES PEA-PATHOGENICITY-RELATED GENES ALSO CARRIES A TRAIT FOR PEA RHIZOSPHERE COMPETITIVENESS

Results presented in this appendix showed that *N. haematococca* field isolates pathogenic on pea could use homoserine as a nitrogen and carbon growth substrate while isolates from other hosts and habitats usually could not. These studies also demonstrated that the gene(s) conferring the ability to grow on homoserine are located on the same CD chromosome that contains the Pea Pathogenicity (*PEP*) cluster. My research contributions to this research consisted of real-time PCR analyses showing that a portion of the *PDA1* CD chromosome containing the gene(s) for homoserine utilization conferred a competitive advantage for growth in the pea rhizosphere (Figure 5) and Southern hybridizations showing that Tr78.2 contained a portion of the *PDA1* CD chromosome (Figure 4). The homoserine growth assays (Tables 1, 2, and 3, Figure 1) were performed by Dr. M. Rodriguez-Carres, the Pulsed Field Gel Electrophoresis analysis (Figure 2) was performed by Dr. M. Taga, interphase nuclei hybridization confirming the translocation (Figure 3) was performed by Dr. D. Tsuchiya, and selection of the homoserine deficient isolates of *N. haematoccoca* by benomyl treatment was performed by Dr. M. Rodriguez-Carres in collaboration with H. Tran.

APPENDIX B: THE EFFECT OF CONDITIONALLY DISPENSABLE CHROMOSOMES OF *NECTRIA HAEMATOCOCCA* MATING POPULATION VI (MPVI) ON RHIZOSPHERE COLONIZATION OF SOYBEAN, TOMATO, DIPLOID ALFALFA, AND PEA

Previous studies have shown that habitat-determining genes can be found on supernumerary chromosomes called "conditionally dispensable" (CD) chromosomes in the habitat-diverse fungus *Nectria haematococca* MPVI and that the *PDA1* CD chromosome gave this fungus a competitive advantage in the pea rhizosphere. Results presented in this appendix showed that both the *PDA1* and *PDA6* CD chromosomes confer a competitive advantage in the rhizosphere of soybean, whereas only the *PDA6* CD chromosome confers a competitive advantage in the rhizospheres of tomato and diploid alfalfa, and only the *PDA1* CD chromosome confers a competitive advantage in the rhizosphere of pea. All of the research in this appendix is mine except designing of actin primers and probe, which was done by Dr. X. Liu.

APPENDIX C: IDENTIFICATION OF HOMOSERINE UTILIZATION GENES IN *NECTRIA HAEMATOCOCCA* MATING POPULATION VI (MPVI) AND THEIR CONTRIBUTION TO RHIZOSPHERE COMPETENCY

In Appendix A of this work it was shown that the fungal plant pathogen *Nectria haematococca* MPVI can utilize homoserine as a sole carbon and nitrogen source and that the gene(s) for this trait is on a conditionally dispensable (CD) chromosome. I showed that the *PDA1* CD chromosome that contains the gene(s) conferring the HS-utilization phenotype also gave *N. haematococca* MPVI an increased competitive

advantage in the pea rhizosphere. In this appendix I used a combination of fungal transformation, insertional mutagenesis, and bioinformatics analyses to specifically locate and identify the genes on the *Nectria haematococca* MPVI *PDA1* CD chromosome that are responsible for the HS-utilization (HUT) phenotype and real-time PCR analyses to determine if the identified genes contribute to an increased competitive ability of *Nectria haematococca* in the rhizosphere of pea plants. All of the research in this appendix is mine except for the following: the cosmid clone library was prepared by Dr. M. Rodriguez-Carres, the determination of codon usage bias, which was done by Dr. J. Coleman, cosmid clone end-sequencing, which was done by the Arizona Genomics Institute, and the sequencing of the genome of *N. haematococca*, which was done by the Department of Energy Joint Genomic Institute (DOE-JGI).

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APPENDIX A: THE SUPERNUMERARY CHROMOSOME OF *NECTRIA HAEMATOCOCCA* THAT CARRIES PEA-PATHOGENICITY-RELATED GENES ALSO CARRIES A TRAIT FOR PEA RHIZOSPHERE COMPETITIVENESS

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To whom it may concern:

I wish to include a copy of the article "The supernumerary chromosome of *Nectria haematococca* that carries pea pathogenicity genes also carries a trait for rhizosphere competitiveness on pea" (AEM00351-08), which has been accepted for publication in Applied and Environmental Microbiology, in my PhD dissertation. I am a co-first author on this paper, and this article contains part of the research I performed during my dissertation. Please advise me on how to proceed with this request.

Sincerely,

Gerard J. White
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The Supernumerary Chromosome of *Nectria haematococca* That Carries Pea-Pathogenicity-Related Genes Also Carries a Trait for Pea Rhizosphere Competitiveness▽

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Fungi are found in a wide range of environments, and the ecological and host diversity of the fungus *Nectria haematococca* has been shown to be due in part to unique genes on different supernumerary chromosomes. These chromosomes have been called "conditionally dispensable" (CD) since they are not needed for axenic growth but are important for expanding the host range of individual isolates. From a biological perspective, the CD chromosomes can be compared to bacterial plasmids that carry unique genes that can define the habits of these microorganisms. The current study establishes that the *N. haematococca PDA1*-CD chromosome, which contains the genes for pea pathogenicity (*PEP* cluster) on pea roots, also carries a gene(s) for the utilization of homoserine, a compound found in large amounts in pea root exudates. Competition studies demonstrate that an isolate that lacks the *PEP* cluster but carries a portion of the CD chromosome which includes the homoserine utilization (*HUT*) gene(s) is more competitive in the pea rhizosphere than an isolate without the CD chromosome.

Plants release as much as 40% of their photosynthates into the soil through their roots (52). The excreted photosynthetic products have various functions, such as detoxifying soil toxins, sequestering nutrients, lubricating the roots for growth through soil, signaling between adjacent plants and between plants and beneficial microbes, and supporting the growth of different types of microbes in the rhizosphere (16). The phenomenon whereby microbial populations are increased in soil that is under the direct influence of root exudates is known as the "rhizosphere effect" (4). Most researchers agree that there is an enrichment of a specific spectrum of microorganisms in the rhizosphere and that this population is less diverse than that in the bulk soil, which is not under the direct influence of root exudates (4, 16). Less accepted are how often a specific microbial genotype is favored in the rhizosphere by a specific plant species or genotype and whether there are particular traits of plants and microbes that control this specificity.

Over the past ~20 years, experiments combining the genetic dissection of rhizosphere colonization with other novel approaches have identified several bacterial traits for rhizosphere competence and colonization (23, 28, 34). For example, studies on the symbiotic bacteria that fix nitrogen in legume roots have demonstrated that the *NOD* genes, which induce nodulation of plant roots in response to specific plant signals, are found on the symbiosis (Sym) plasmid (25). Sym plasmids carry not only the genes for nodulating specific hosts (36) but also the genes for catabolizing unique compounds present in the root exudates of the host plants (1, 10). The correlation between nodulation specificity and the ability to catabolize specific host root exudates was originally suggested by VanEgeraat (45), who discovered that the pea-nodulating bacterium *Rhizobium leguminosarum* could catabolize homoserine (HS), a compound found in high concentrations in pea root exudates. It was shown later that the Sym plasmid of *R. leguminosarum* carries the gene for HS utilization as well as the *NOD* genes (6).

Although the diversity of fungi in the rhizosphere is well recognized, in contrast to the case for bacteria the genetic determinants that enable fungi to inhabit the rhizosphere of host plants remain, for the most part, unknown. Fungal interactions with plant roots are of major ecological and economic importance for the development of mycorrhizal symbiosis and the control of soilborne pathogens. In the current study, the understanding of the bacterial genes involved in symbiosis and in the colonization of the host rhizosphere (23, 28, 34) was used as a model to identify some of the genes that may play parallel roles in the root-pathogenic fungus *Nectria haematococca* (anamorph, *Fusarium solani*).

The fungus *N. haematococca* is found as a soil saprobe, a commensal organism in the rhizosphere, and a pathogen of many different plant species (26, 46). The genetic and habitat diversity of *N. haematococca* is due in part to the presence of supernumerary chromosomes (49). These "extra" chromosomes are called "conditionally dispensable" (CD) chromosomes because while they are not required for axenic growth, they may allow isolates to have an expanded host range (3). There are several different CD chromosomes, one of which, the *PDA1*-CD chromosome, carries a cluster of genes for pea pathogenicity (*PEP* cluster) (13). *N. haematococca* isolates

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TABLE 1. Growth on HS of *N. haematococca* isolates that are pathogenic on pea plants[a]

Isolate	Dry wt (mg)[c]	Radial growth (mm)[d]	Geographical origin
T1	6.5	12/20	New York
T2[b]	6.5	24/24	New York
T8[b]	5.7	12/15	New York
T9[b]	6.8	18/18	New York
T10	5.7	18/20	New York
T17	5.2	15/20	New York
T23[b]	7	ND	New York
T30[b]	4.7	12/20	Washington
T63	5.6	20/25	New York
T70[b]	6.9	20/20	Michigan
T468[b]	7.3	17/14	England
T547[b]	6.9	18/18	New Zealand
T406	5.6	14/15	Taiwan
T558[e]	4.6	22/21	Japan
T559[e]	4	20/25	Japan
T560[e]	4.8	20/25	Japan
T561[e]	3.6	26/25	Japan

[a] Pathogenicity data are in references 9, 41, 46, 47, and 48 or are unpublished data.
[b] Isolates which are known to have a *PDA1*-CD chromosome, based on the presence of an ~1.6-Mb chromosome containing the *PDA1* gene, as determined by PFGE and/or Southern analysis (8, 27, 41).
[c] After growth in 2 ml of 25-mg/ml L-HS for 14 days. Isolates grown on 2 ml of 25-mg/ml L-glutamic acid had dry weights ranging from 4.3 to 4.8 mg.
[d] Diameter of the mycelial colony after growth for 4 days on semisolid medium containing 25 mg/ml of L-HS (first number) or 10 mg/ml of glucose (second number). ND, not determined.
[e] Unpublished data.

TABLE 2. Growth on HS of *N. haematococca* field isolates that are not pathogenic on pea plants[a]

Isolate	Dry wt (mg)[b]	Radial growth (mm)[c]	Geographical origin	Source
T34[d]	0.5	0/15	Australia	Cooling tower
T77[d]	0.1	0/23	Pennsylvania	Alfalfa
T78[d]	0.4	0/20	Pennsylvania	Alfalfa
T95[d]	0.5	0/25	South Carolina	Tulip tree
T110[d]	0.4	ND	Mississippi	Cottonwood
T213[d]	0	ND	Kentucky	Cottonwood
T215[d]	0.4	0/13	North Dakota	Potato
T217[d]	0.5	0/25	Pennsylvania	Carnation
T219[d]	0	0/15	New York	Soil
T272[d]	5.1	10/20	Utah	Unknown
T273[d]	4.7	16/20	Utah	Unknown
T288[d]	1.3	0/25	Pennsylvania	Soil
T300[d]	0.7	0/20	Pennsylvania	Red clover
T314[d]	0.8	0/22	Pennsylvania	Shrimp
T347[d]	0.1	0/15	New York	Alfalfa
T351[d]	0.6	0/16	New York	Alfalfa
T386[d]	5.2	ND	Spain	Chickpea
T562	0.6	0/20	Japan	Alfalfa
T474[d]	0.4	0/23	England	Pea soil

[a] Pathogenicity data are in references 27, 41, 46, 47, and 48 or are unpublished data.
[b] After growth in 2 ml of 25-mg/ml L-HS for 14 days. Isolates grown in 2 ml of 25-mg/ml L-glutamic acid had values ranging from 4.3 to 4.8 mg.
[c] Diameter of the mycelial colony after growth for 4 days on semisolid medium containing 25 mg/ml of L-HS (first number) or 10 mg/ml of glucose (second number). ND = not determined.
[d] Isolates without the *PDA1*-CD chromosome, based on PFGE and/or Southern hybridization analysis (8, 27, 41).

with the *PDA1*-CD chromosome are highly virulent on pea plants (3, 27, 49). The *PDA1* gene, from which the *PDA1*-CD chromosome takes its name, is a member of the *PEP* cluster and codes for a cytochrome P450 enzyme that detoxifies the pea phytoalexin (defense molecule) pisatin (24). The *PDA1* gene is used routinely as a marker for the presence of this CD chromosome (49).

In this study, we show that isolates of *N. haematococca* that are pathogenic on pea plants can grow on HS as a sole carbon and nitrogen source but that isolates from other hosts and sources usually cannot. Furthermore, we show that the gene(s) for HS utilization (*HUT*) is on the *PDA1*-CD chromosome. We also report the development of a real-time PCR technique that overcomes one of the major hurdles in studying fungal rhizosphere competence and colonization, i.e., finding an accurate means to quantify fungal biomass (cell number) in the rhizosphere (32). Using this technique, we demonstrate that the portion of the *PDA1*-CD chromosome that contains the *HUT* gene(s) provides *N. haematococca* isolates with a competitive advantage in the pea rhizosphere.

MATERIALS AND METHODS

Fungal strains. Isolates of *N. haematococca* were obtained from the culture collection of Hans VanEtten (Tables 1, 2, and 3). Stock cultures of *N. haematococca* were maintained as slant cultures on V-8 agar medium (M-29 medium) (39). Cultures were grown in the dark at 27°C.

Media. Measurements of the growth of *N. haematococca* on different carbon and/or nitrogen sources were carried out on solidified *Ustilago* minimal medium (M-100 medium) (39). M-100 medium, which contains 10 mg/ml glucose as the carbon source and 3 mg/ml $NH_3(NO_3)_2$ as the nitrogen source, was modified by replacing glucose and/or $NH_3(NO_3)_2$ with the following chemicals: HS (Sigma-Aldrich, St. Louis, MO) (HS medium), trigonelline (Sigma-Aldrich, St. Louis, MO) (TGR medium), γ-glutamyl-D-alanine (a gift from Andrew Mort, Department of Biochemistry, Molecular and Cellular Biology, Oklahoma State University) (GAA medium), and glutamic acid (Sigma-Aldrich, St. Louis, MO). In some experiments, DL-HS was used instead of L-HS because DL-HS was more readily available. None of the isolates could grow on D-HS.

Spore production and collection. *N. haematococca* spores were produced in petri dishes containing solidified V-8 agar (39). Cultures were incubated at 24°C ± 1°C under lighted conditions to encourage conidiation. After 1 to 2 weeks, the spores were harvested, rinsed with sterile water, suspended in water, and counted. For rhizosphere competence assays, the numbers of viable spores in the inocula were confirmed by dilution plating the spore suspensions on potato dextrose agar (Difco Laboratories, Detroit, MI) and counting fungal colonies.

Growth assays. To measure growth as the change in culture turbidity, 100 μl of liquid medium containing the substrate being tested was placed into each well of a 96-well microtiter plate, and each well was inoculated with ~500 spores. Plates were incubated in a moist chamber at 27°C. Turbidity was measured with a spectrophotometer (OpsysMR; ThermoLabsystem) as the optical densities at 540 nm and 620 nm at 12- to 24-h intervals for 5 days. To assay growth by measuring the change in dry weight, test tubes containing 2 ml of liquid medium were inoculated with 5×10^3 to 10×10^3 conidia and incubated at room temperature on a gyrorotatory shaker at 100 rpm for 13 days. Mycelium was collected by filtration, dried, and weighed. To assay radial growth, semisolid media containing Gelrite Gellan gum (3.5 mg/ml; Sigma-Aldrich, St. Louis, MO) as the solidifying agent and either 0.01 mg/ml glucose or 25 mg/ml L-HS were inoculated with 25 μl of a spore suspension containing 1,000 to 5,000 conidia. Colony diameters were measured after 4 days.

Benomyl treatment to induce loss of the *PDA1*-CD chromosome. Fifty milliliters of M-100 medium containing 37 μg of benomyl/ml was inoculated with ~3×10^4 spores of isolate 77-13-4 and incubated at room temperature with shaking at 190 rpm. After 8 days, the culture was filtered through two layers of cotton filter paper, and fresh liquid M-100 and benomyl were added to the filtrate to obtain a final volume of 75 ml with 37 μg of benomyl/ml. After another 6 days, the culture was filtered and the filtrate was centrifuged to collect the spores. The spores were washed three times with sterile water and used to inoculate 50 ml of liquid DL-HS medium (50 mg DL-HS/ml). The cultures were incubated overnight at room temperature with shaking at 50 rpm. The culture was then filtered through one layer of cotton filter paper to remove germinated spores, and the

TABLE 3. Growth on HS of selected isolates of *N. haematococca* that do or do not contain the *PDA1*-CD chromosome or its marker gene, *PDA1*

Isolate(s)	Description	Growth on HS[h]
77-1-3, 77-5-5, 77-13-1, 94-6-1, 44-100,[a] 44-1, 44-22, 44-36, 44-46, 44-64, 44-75	Meiotic progeny without the *PDA1* gene[g]	−
77-1-3, 77-5-7, 77-13-4,[b] 77-13-7,[b] 44-16, 44-20, 44-37, 94-1-6	Meiotic progeny with the *PDA1* gene[g]	+
B-13, B-32, B-33, B-34, B-35, B-36, B-37	*hph*-tagged CD chromosome loss induced by benomyl treatment of Tr18.5 and isolates selected by loss of hygromycin resistance[c]	−
HT1, HT3, HT4, HT5, HT6, HT7, HT8, HT9, HT10	CD chromosome loss induced by benomyl treatment of 77-13-4 and isolates selected by loss of HS utilization	−
Tr115.1,[d] Tr135.8,[d] Tr23.1[d]	CD chromosome lost during transformation of 77-13-4[e]	−
Tr78.2[d]	Portion of the CD chromosome was retained after transformation of 77-13-4[e]	+
N-15	100-kb truncation of the CD chromosome in 77-13-7[f]	+
Tr18.5[d]	*PDA1* gene disruptant of 77-13-7[e]	+
Tr86.1[d]	*PDA1* gene disruptant of 77-13-4[e]	+

[a] Lack of *PDA1*-CD chromosome demonstrated (8).
[b] Presence of *PDA1*-CD chromosome demonstrated (51).
[c] Isolates shown to lack a *PDA1*-CD chromosome (50).
[d] Absence of *PDA1*-CD chromosome demonstrated (51).
[e] Isolates from reference 51.
[f] Isolate from reference 18.
[g] Isolates from reference 17.
[h] Growth was determined as described in the legend to Fig. 1. +, obvious growth after 4 days; −, no growth after 4 days on semisolid medium containing 25 mg/ml of L-HS.

filtrate was centrifuged to collect the ungerminated spores. The spores were suspended in water and counted, and 150 spores were spread onto M-100 agar containing 0.8 mg/ml Triton X-100 (Fisher Scientific, Pittsburgh, PA). The plates (100 × 15 mm) were overlaid immediately with nylon membranes (curtain fabric; 3 fibers/mm) as described previously (50). After 24 h, the nylon membranes were transferred to HS agar (50 mg DL-HS/ml) containing 0.4 mg/ml Triton X-100. Spores were collected from those colonies on the M-100 agar that did (HUT^+) and did not (HUT^-) grow on HS agar. After single-spore isolation, the HUT phenotype of a single spore culture from each of the colonies was retested on HS agar.

Southern hybridization. For Southern hybridization, 1 μg of genomic DNA was digested with XhoI according to standard protocols. The digested DNA was size fractionated by electrophoresis on a 0.7% agarose gel, followed by transfer to a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech Ltd.). The membranes were prehybridized for 4 h at 42°C in prehybridization solution (50% formamide, 5× Denhardt's solution, 1 M NaCl, 50 mM PIPES, 0.5% Sarkosyl, 500 μg/ml salmon sperm DNA, 25 μg/ml tRNA, and 10 mM EDTA) and then hybridized overnight with one of the probes at 42°C.

To obtain DNA templates for the synthesis of radioactively labeled probes, three cosmids (3B04, 6H10, and 8A10) from a *PDA1*-CD chromosome-specific library were digested with either EcoRI or EcoRI and EcoRV. The restriction fragments were resolved by electrophoresis in 1% low-melting-point agarose, all at 24 V/cm overnight. The portions of the gel containing a 1.7-kb EcoRI fragment from cosmid 3B04, a 1.5-kb EcoRI fragment from cosmid 6H10, and a 1.5-kb EcoRI/EcoRV fragment from cosmid 8A10 were excised, and the DNAs were radiolabeled using a Rad-Prime kit (Gibco-BRL) according to the manufacturer's instructions.

Following hybridization, membranes were washed twice in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) at room temperature for 30 min, twice in 2× SSPE containing 0.1% sodium dodecyl sulfate at 65°C for 30 min, and twice in 0.2× SSPE containing 0.1% sodium dodecyl sulfate at 65°C for 30 min.

PFGE analyses of chromosomal DNA. The preparation of protoplasts for karyotypic chromosomal analysis was performed as described previously by Taga et al. (40), with the exception that 1.2 M $MgSO_4$ was used as the osmotic medium instead of 1.2 M NaCl. Chromosomes were resolved in agarose gels (0.008 g/ml pulsed-field certified agarose; Bio-Rad Laboratories, Inc., Hercules, CA) prepared in running buffer (0.5× Tris-borate-EDTA) by pulsed-field gel electrophoresis (PFGE).

Fluorescence in situ hybridization. Fixed specimens of mitotic nuclei and chromosomes for cytological observation and hybridizations were prepared as previously described (40, 43). The probe, genomic DNA from isolate 77-13-4, was labeled with biotin-14-dATP by nick translation using the BioNick labeling system (Invitrogen Corp., Carlsbad, CA). Observations were made with an Olympus BX60 epifluorescence microscope (Olympus America Inc., Center Valley, PA) equipped with an Olympus U-MWU2 excitation cube (Olympus America Inc.) for DAPI (4′,6′-diamidino-2-phenylindole), an Olympus U-MNB excitation cube (Olympus America Inc.) for Alexa Fluor 488, and a triple-band-pass filter (Chroma Technology Corp., Rockingham, VT) for DAPI and Alexa Fluor 488. Photographs were taken with an Optronics DEI-750D charge-coupled device camera (Optronics, Goleta, CA).

Rhizosphere competition assays. A replacement series technique (5) was used to measure rhizosphere competition as a function of the relative abundances of the two competing *N. haematococca* isolates. Pea seeds were surface sterilized in 70% ethanol for 5 min and in a 2.5% sodium hypochlorite solution for 10 min and then rinsed thoroughly with sterile distilled water. Surface-sterilized seeds were soaked in sterile distilled water overnight at room temperature to allow the seeds to imbibe and then were planted (one seed per box) in Magenta GA-7 boxes (Magenta Corp., Chicago, IL) containing 100 g of sterile potting mixture (4 parts vermiculite to 1 part quartz sand wetted with 1 liter of sterile distilled water per 10 liter of mixture). After germination, plants were grown under a 12-hour–12-hour light-dark regimen at 24°C ± 1°C. After a 2-week growth period, the potting mixture was inoculated with spores of two isolates (Tr78.2 and either HT1 or HT5) prepared at a constant density of 10^5 *N. haematococca* spores per gram of potting mixture but at various ratios of the two isolates (0:100, 25:75, 50:50, 75:25, and 100:0). The plants were grown for another 2 or 3 weeks under the same conditions. During the growth period, the plants were watered with Hoagland's solution every 4 to 6 days. At the end of the 2- or 3-week period, the rhizospheres and roots were harvested by cutting the stem immediately above the uppermost roots and gently shaking off the loose potting mixture. The harvested roots and adhering potting mixture were lyophilized and ground to a fine powder in liquid nitrogen by use of a mortar and pestle. DNA for real-time PCR was extracted from 1 gram of ground rhizosphere material by use of an Ultraclean Soil DNA kit (Mo Bio Laboratories, Inc., Solana Beach, CA). Six replicates were performed for each ratio of *N. haematococca* isolates.

deWit replacement curves. Competition between HUT^+ and HUT^- isolates is shown diagrammatically by plotting the results on a deWit replacement curve, on which dashed lines represent the growth of the isolates without competition and solid lines represent the actual growth under competition. If there is no competition between the two isolates, i.e., each can colonize the rhizosphere equally, then the ratios of the two isolates recovered from the rhizospheres of plants inoculated with HUT^+/HUT^- mixtures should be the same as the inoculation ratios.

Real-time quantitative reverse transcription-PCR. The number of cells in each sample was determined from the target gene copy number, which was quantified by comparing the cycle threshold (C_T) value of the samples to the C_T value of the respective standard curve. Standard curves were constructed with serial dilutions of the PCR products obtained from the genomic DNAs of *N. haematococca* isolate 77-13-4 and the pea plant (cv. Little Marvel), using the same primer pairs as those used for real-time quantitative PCR with rhizosphere samples. The sequence for the *N. haematococca* actin gene was obtained from Liu et al. (22) and used for real-time PCR to determine the total number of fungal cells of isolates Tr78.2 and HT1 in the rhizosphere samples. A portion of the *N. haematococca PDA1* sequence was used for real-time PCR to detect the number of fungal cells of Tr78.2. Tr78.2 lacks a wild-type copy of *PDA1* but contains the hygromycin resistance gene (*hph*) flanked by 692 bp 5′ and 888 bp 3′ of the *PDA1* gene (51). The *PDA1* sequence from the 888-bp 3′-flanking region was used for real-time PCR. Real-time quantitative PCR using TaqMan technology was performed on an Abbott Prism system (Abbott Park, IL) according to the manufacturer's protocol. Sequences of the primers (Invitrogen Corporation, Carlsbad, CA) and TaqMan fluorescent probes (Applied Biosystems, Foster City, CA) used in the quantitative real-time PCR study were as follows: *PDA1* forward primer, 5′-GATGAGCAGACTGAGGTTGGT3′; *PDA1* reverse primer, 5′-CTGTGATGCCAAGGTCACTTA-3′; and *PDA1* probe, 6-carboxyfluorescein (FAM)-AAGCGATCTTTGGCAACGATGCAAG-6-carboxytetramethylrhodamine (TAMRA); actin gene forward primer, 5′-ATCCACGTCAC CACCTTCAA-3′; actin gene reverse primer, 5′-GTGCCAGAGTTAGAAATG ATC-3′; and actin gene probe, FAM-ACATCGACATCACACTTCATGATGG AG-TAMRA.

The rubisco activase (*RCA*) gene from the pea was used to measure the number of pea cells in the sample and to normalize the amount and quality of the genomic DNA. *RCA* mRNA sequences were obtained from the GenBank sequence database for 12 plant species and aligned using the Clustal function of MacVector software. The region with the highest identity across species was used to design the forward (5′-CATTATGATGAGTGCTGGAGA-3′) and reverse (5′-TCCATACGACCATCACGGAT-3′) PCR primers. These primers were used to amplify the corresponding, ~350-bp region in the pea *RCA* gene, using genomic DNA of the pea as the template. The pea *RCA* DNA sequence was used similarly to design the PCR primers and the TaqMan probe. The following were used for *RCA*: forward primer, 5′-CCTCTTCATCAACGATCTCGAT-3′; reverse primer, 5′-GGTTGTCAGCAATGTTCATGAG-3′; and probe, tetrachloro-6-carboxyfluorescein (TET)-CACCGTCAACAACCAGATGGTGA-ATG-TAMRA.

Each TaqMan probe was designed to anneal to a specific sequence between the forward and reverse primers of its target gene and to have at least a 5°C higher melting temperature than that of the PCR primers. Individual probes contained a reporter fluorochrome (FAM for *PDA1* and the actin gene and TET for *RCA*) at the 5′ end and a quencher fluorochrome (TAMRA) at the 3′ end. PCR analyses were performed in duplicate, with duplicate reactions on each run. Each reaction mix had a total volume of 25 μl containing 12.5 μl of qPCR Mastermix Plus (contains reaction buffer deoxynucleoside triphosphates [including dUTP], Hot Goldstar DNA polymerase, 5 mM $MgCl_2$, uracil-*N*-glycosylase, stabilizers, and a passive reference; Invitrogen Corp., Carlsbad, CA), 300 nM forward primer, 300 nM reverse primer, 200 nM TaqMan probe, 2 μl rhizosphere DNA template, and 9 μl of sterile distilled water. The PCR parameters were as follows: an initial uracil-*N*-glycosylase step at 50°C for 2 min, followed by an initial denaturation and Hot Goldstar DNA polymerase activation step at 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 30 s. Real-time PCRs for *PDA1*, the actin gene, and pea *RCA* were performed at least three times.

RESULTS

Growth of *N. haematococca* on compounds found in pea root exudates. Previous studies have shown that all isolates of *N. haematococca*, independent of their pathogenicity and CD chromosome content, can grow on pea root exudates (11). In the present study, chemicals which are found in pea root exudates (19), i.e., HS, GAA, and TGR, were tested for the ability to support the growth of *N. haematococca*. Growth on HS and TGR was measured as an increase in biomass in liquid media and as an increase in colony diameter on semisolid media. Due to the small amounts of compound available, growth on GAA was measured only by the increase in turbidity.

All four isolates (T77, T219, T347, and 77-13-4) used in an initial screen were able to use GAA as a sole C and N source. However, only 77-13-4, the sole isolate of the four that is a pathogen on pea plants and carries the *PDA1*-CD chromosome (51), could grow on HS medium. TGR was inhibitory to growth when it was added to M-100 medium at concentrations above 0.05%, and no isolate could use this compound as a sole C and/or N source (data not shown).

Relationship between an isolate's pathogenicity and its ability to use HS. To test further whether the pathogenicity of a field isolate is correlated with its ability to use HS as a sole C and N source, 36 field isolates from different hosts and geographic locations were examined. The 17 field isolates in Table 1 are pathogenic on pea plants, and some are known to contain a *PDA1*-CD chromosome based on Southern hybridization and PFGE analyses (27, 41). All of these isolates grew on HS, regardless of their geographic origin (Table 1). None of the 19 isolates in Table 2 are pathogenic on pea plants, and although a few of them, based on PFGE and Southern hybridization analyses, contain an ~1.6-Mb chromosome, they do not contain a *PDA1* gene (27). The isolates in Table 2 were obtained from a variety of habitats other than pea plants, and only three (T272, T273, and T386) were able to use HS as the sole C and N source (Table 2). Isolates T272 and T273 were obtained from soils with unknown plant associations. Isolate T386 came from a chickpea plant. All of the isolates in Tables 1 and 2 could grow on glutamic acid as a sole C and N source, whereas pea-pathogenic isolates had an additional metabolic capability, i.e., the ability to use the amino acid HS as a sole C and N source.

Location of the HS utilization (*HUT*) gene(s) on the *PDA1*-CD chromosome. Previous studies have shown that the CD chromosomes of *N. haematococca* can be lost during sexual crosses, during transformation, and following treatment with benomyl (49, 50, 51). To determine if *HUT* is on the *PDA1*-CD chromosome, a series of related isolates, which differed with respect to the presence of the *PDA1*-CD chromosome or its marker gene, *PDA1*, were examined for HS utilization. A total of 18 progeny from three different crosses (crosses 44, 77, and 94) (17) in which *PDA1* segregated were assayed for the HUT phenotype. The 8 progeny that inherited *PDA1* grew on HS, whereas the 11 progeny that did not inherit *PDA1* did not grow on HS (Table 3; Fig. 1). These results further support the hypothesis that the *PDA1* gene and the gene(s) conferring the ability to grow on HS are located in the same linkage group, i.e., the *PDA1*-CD chromosome.

This linkage was also tested with isolates that had lost the *PDA1*-CD chromosome during experimental manipulations. It had been observed that the *PDA1*-CD chromosome of *N. haematococca* isolate 77-13-4 was occasionally lost during transformation (51). Three of the four transformants of 77-13-4 (Tr115.1, Tr135.8, and Tr23.1) that had lost the *PDA1*-CD chromosome (51) could not grow on HS (Table 3; Fig. 1). Kistler et al. (18) also generated a transformant of 77-13-7, transformant N15, which lacked the *PEP* cluster due to an ~100-kb truncation in the *PDA1*-CD chromosome. However, transformant N15 can grow on HS, indicating that the *HUT* gene(s) is located on the remaining portion of the *PDA1*-CD chromosome and is not part of the previously characterized *PEP* cluster.

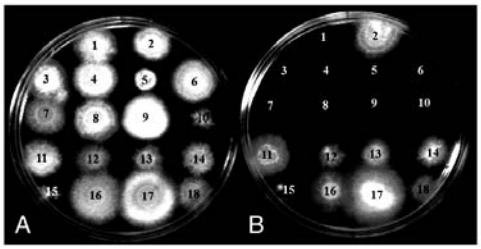

FIG. 1. Growth of *N. haematococca* isolates on glucose (A) and HS (B) media. Isolates lacking the *PDA1*-CD chromosome or its marker gene, *PDA1*, included the following: 1, isolate Tr23.1; 2, isolate Tr78.2; 3, isolate Tr115.1; 4, isolate Tr135.8; 5, isolate B-13; 6, isolate 44-100; 7, isolate 44-75; 8, isolate 44-22; 9, isolate 44-1; and 10, isolate 94-6-1. Isolates with the *PDA1*-CD chromosome or its marker gene, *PDA1*, included the following: 11, isolate Tr86.1; 12, isolate 77-2-3; 13, isolate 77-13-4; 14, isolate 77-13-7; 15, isolate N15; 16, isolate 44-16; 17, isolate 44-37; and 18, isolate 94-1-6. Plates were photographed 4 days after inoculation.

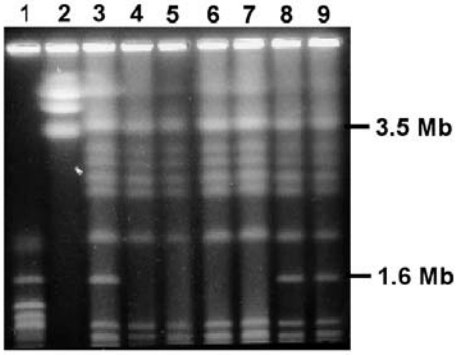

FIG. 2. PFGE karyotypes of isolate 77-13-4 and of mutants of 77-13-4 that lost the ability to grow on HS after treatment with benomyl. Lane 1, chromosomes of *Saccharomyces cerevisiae*; lane 2, chromosomes of *Saccharomyces pombe*; lane 3, chromosomes of 77-13-4; lanes 4 to 7, chromosomes of isolates that were HUT^- (lane 4, HT1; lane 5, HT3; lane 6, HT4; and lane 7, HT5); lanes 8 and 9, chromosomes of isolates that were HUT^+ after benomyl treatment (lane 8, HT11; and lane 9, HT12).

Previous studies evaluating the role of *PDA1* in pea pathogenicity used site-directed mutagenesis to disrupt the *PDA1* gene in isolate 77-13-7 (51). The resulting PDA-negative (PDA^-) transformants had the *PDA1*-CD chromosome tagged with a hygromycin resistance gene (51) and were HUT^+ (Table 3). One of these transformants (Tr18.5) was treated with benomyl, which can cause aneuploidy during vegetative growth in fungi, and the loss of the CD chromosome (50) was detected by a loss of hygromycin resistance. All seven hygromycin-sensitive isolates identified in those experiments (B-13, B-32, B-33, B-34, B-35, B-36, and B-37) lack the *PDA1*-CD chromosome (50) and cannot grow on HS (Table 3; Fig. 1). A similar benomyl treatment was performed on 77-13-4 in the current study, but in this case isolates were selected for a loss of the ability to grow on HS. Nine HUT^- isolates were obtained (Table 3), and the chromosomes of four (HT1, HT3, HT4, and HT5) were resolved by PFGE; all had lost the *PDA1*-CD chromosome (Fig. 2). As controls, two isolates (HT11 and HT12) that retained the ability to grow on HS after the benomyl treatment were also analyzed and shown to have the *PDA1*-CD chromosome (Fig. 2).

Isolate Tr78.2 contains the portion of the *PDA1*-CD chromosome that confers *HUT*. The only result that appears to conflict with the hypothesis that the *HUT* gene(s) is on the *PDA1*-CD chromosome is that transformant Tr78.2, which was shown by Southern analysis to lack a wild-type *PDA1* gene and by PFGE analysis to lack the *PDA1*-CD chromosome (51), grew on HS (Table 3; Fig. 1). However, it is possible that the translocation of a portion of the *PDA1*-CD chromosome occurred during transformation and was missed in the previous study, since it is difficult to resolve chromosomes larger than ~4 Mb by PFGE (49).

To determine whether a portion of the *PDA1*-CD chromosome might have been retained in isolate Tr78.2, interphase nuclei from Tr78.2 were examined using fluorescence in situ hybridization (Fig. 3). Nuclei from Tr78.2 were hybridized first with genomic DNA from isolate HT1 and subsequently with biotin-labeled DNA from isolate 77-13-4. This type of experiment has previously been shown to detect CD chromosome-specific DNA in isolates that differ only in the presence of a CD chromosome (40). A strongly hybridizing signal was detected (Fig. 3), which is consistent with Tr78.2 carrying the portion of the *PDA1*-CD chromosome that contains the *HUT* gene(s).

To verify that Tr78.2 contained a portion of the *PDA1*-CD chromosome, Southern blots of Tr78.2 and other isolates, with

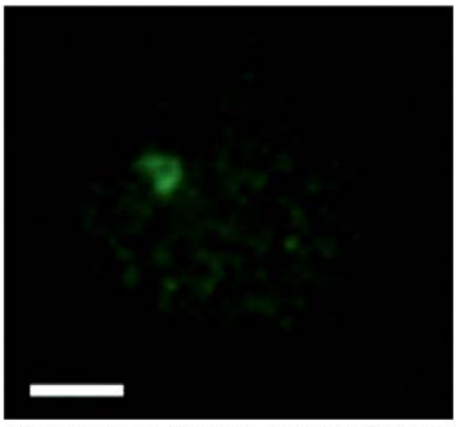

FIG. 3. Visualization of interphase chromosomes of isolate Tr78.2. Interphase nuclei of Tr78.2 were hybridized with genomic DNA from isolate HT1, followed by hybridization with biotin-labeled DNA from isolate 77-13-4. The biotin-labeled DNA was detected with goat anti-biotin antibody, followed by staining with Alexa Fluor 488-conjugated rabbit anti-goat antibody. Scale bar = 2 μm.

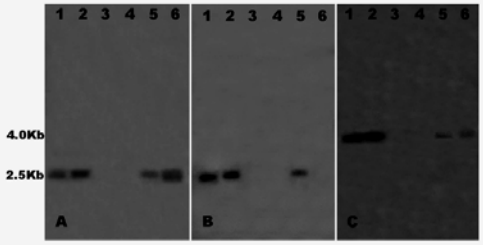

FIG. 4. Southern hybridization analysis of DNAs from isolates 77-13-4, 77-13-7, HT1, HT5, and Tr78.2 (lanes 1 to 5, respectively) for the presence of an ~800-kb portion of the *PDA1*-CD chromosome. Fragments used as probes were a 1.7-kb EcoRI fragment from cosmid 3B04, which contains DNA from one end of the 800-kb region (A); a 1.5-kb EcoRI fragment from cosmid 6H10, which contains DNA from an internal portion of the 800-kb region (B); and a 1.5-kb EcoRI/EcoRV fragment from cosmid 8A10, which contains DNA from the opposite end of the 800-kb region (C).

and without the *PDA1*-CD chromosome, were probed with DNAs from cosmid clones from the *PDA1*-CD chromosome. A previously constructed partial physical map of the *PDA1*-CD chromosome (35) allowed the selection of cosmid clones spanning ~800 kb of the *PDA1*-CD chromosome. DNA fragments from cosmid clones (3B04 and 8A10) that contain DNA from the ends of this 800-kb region and from a cosmid (6H10) that contains DNA from the interior of the 800-kb region were hybridized to genomic DNAs of Tr78.2, 77-13-4 (the source of Tr78.2), and 77-13-7, another isolate from cross 77 that has the *PDA1*-CD chromosome. These fragments hybridized to Tr78.2, 77-13-4, and 77-13-7 but did not hybridize to HT1 and HT5 (Fig. 4), a result consistent with the presence of a portion of the *PDA1*-CD chromosome in Tr78.2.

Analysis of the rhizosphere competitive ability of isolate Tr78.2. To determine if the portion of the *PDA1*-CD chromosome containing the *HUT* gene(s) confers a competitive advantage in the pea rhizosphere, we used a replacement series approach (5). Pea plants were grown for 2 weeks and then inoculated with different ratios of Tr78.2 and HT1 and of Tr78.2 and HT5. Two (for HT1) or three (for HT5) weeks after inoculation, the rhizosphere (roots with adhering potting mix) was removed and the DNA extracted. Real-time PCR analyses of the single-copy actin gene of *N. haematococca* and of a region of the *PDA1* gene from the vector used to produce Tr78.2 were used to determine the total number of fungal cells and the relative number of Tr78.2 cells, respectively. Real-time PCR analyses of the pea *RCA* gene were used to measure the number of plant cells and to normalize the data. When the inoculum was 100% of either Tr78.2 (HUT^+) or HT1 (HUT^-), the same quantity of fungal cells (2.06 ± 0.32 versus 2.17 ± 0.09 actin genes/*RCA* gene) was present in the rhizospheres of pea plants, indicating that the pea rhizosphere had the same carrying capacity for both isolates (Fig. 5). Therefore, when there is no competition between the two isolates, both isolates would be expected to colonize the rhizosphere equally and to be present at the same relative ratios as those present in the initial inoculum. However, the real-time PCR results showed that 2 weeks after the pea rhizosphere was inoculated with 25:75, 50:50, and 75:25 ratios of Tr78.2 to HT1, the HUT^+ isolate Tr78.2 made up 31%, 60%, and 90% of the total fungal biomass, while the HUT^- isolate HT1 made up only 69%, 40%, and 10% of the fungal biomass (Fig. 5). Repeat experiments with isolates HT1 and HT5 gave similar results, with isolate Tr78.2 accounting for up to 87% of the fungal biomass 2 weeks (for HT1) or 3 weeks (for HT5) after inoculation with a 50:50 mixture of both isolates (data not shown). The inoculated roots did not show any disease symptoms, and fungal DNA was not detected inside the roots, which demonstrates, as expected, that the fungi were not growing inside the roots but in the rhizosphere.

DISCUSSION

A priori, it seems likely that a selective ability to utilize a nutrient in the rhizosphere would benefit a rhizosphere-inhabiting organism. This study shows that the ability of *N. haematococca* to utilize the pea root exudate HS is correlated with its ability to be a pea root pathogen and that the gene(s) for HS

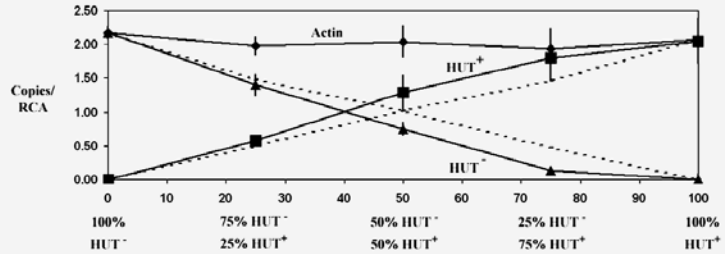

FIG. 5. Relative amounts of isolate Tr78.2 (HUT^+) and isolate HT1 (HUT^-) recovered from the rhizospheres of pea plants 2 weeks after inoculation of 2-week-old seedlings with different ratios of these isolates. Two-week-old pea seedlings were inoculated with spore suspensions containing mixtures (100:0, 75:25, 50:50, 25:75, and 0:100) of Tr78.2 and HT1. A rhizosphere sample was obtained from each treatment (six samples per treatment) 2 weeks after inoculation, and the DNA was extracted. The amounts of the pea *RCA* gene, the *N. haematococca* actin gene, and the 888-bp portion of the *PDA1* gene in each sample were determined by real-time PCR. Broken lines show the expected relative ratios of both isolates if they are not competing. Solid lines show the experimental values for the HUT^+ and HUT^- isolates. The means are significantly different from expected results for the 75:25, 50:50, and 25:75 treatments (Newman-Keuls multiple comparison test; $P < 0.05$).

utilization (*HUT*) is located on the same *PDA1*-CD chromosome as the genes for pea pathogenicity (*PEP* cluster). Furthermore, the portion of the chromosome that contains the *HUT* gene(s) endows an isolate with an increased competitive advantage in the pea rhizosphere. In support of the possibility that the competitive advantage might be due to the *HUT* gene(s), several research groups have engineered plants to produce novel nutrients in their root exudates and have shown that bacteria transformed with the ability to utilize these nutrients have a competitive advantage in the rhizosphere (29, 38). Though not yet tested, one might logically predict that it would also be advantageous for a root pathogen to be more competitive in the rhizosphere prior to its entry into the roots of its host.

The identification on a CD chromosome of another trait which enhances the ability of *N. haematococca* to expand its habitat further supports the notion that fungal CD chromosomes are analogous to host-specifying plasmids in plant-associated bacteria. For example, the different allelic variants of the Ti plasmids in *Agrobacterium tumefaciens* and the *sym* plasmids in *Rhizobium* spp. determine the host specificity for each bacterial strain (2). Furthermore, the symbiotic and nonsymbiotic plasmids of bacteria isolated from the rhizosphere of their host plants often contain genes for the utilization of host-specific root exudates (6, 30, 34). For some bacteria, these utilization genes have been shown to confer an increased competitive ability in the rhizosphere of their respective host plants (15, 34). For example, the *sym* plasmid of the *Sinorhizobium meliloti* bacterium, which nodulates alfalfa roots, carries genes for the catabolism of the alfalfa seed exudate stachydrine (10, 34). These genes for stachydrine catabolism also increase the competitive ability of *S. meliloti* in the rhizosphere of alfalfa (34). In addition, stachydrine not only supports the growth of *S. meliloti* but also induces the expression of its *NOD* genes, which are carried on the *sym* plasmid (33). A parallel situation may exist between the fungus *N. haematococca* and its host, the pea. HS, a root exudate of the pea, supports the growth of pea-pathogenic isolates of this fungus. Others (53) have shown that HS induces the expression of a pectin-degrading gene, *pelD*, which is a pathogenicity gene in this fungus.

The transfer of plasmids between bacteria has long been known to change the properties of the recipient bacterium and is another form of the horizontal transfer of large "genomic islands" of DNA which serves as a major force in the evolution of bacteria, allowing them to inhabit new environments (21). The clustering of genes for the colonization of certain habitats may facilitate their transfer and improve their retention via positive selection in those environments (12, 21). The current work is among the first to provide an example of a specific habitat, the rhizosphere of pea plants, for which the clustering of genes on a CD chromosome might be beneficial not only for host specificity but also for inhabiting an environment conducive to horizontal gene transfer.

Many lines of evidence have implied that all or part of the CD chromosomes might have originated through horizontal gene transfer. First, the *PEP* genes on the *PDA1*-CD chromosome have a different codon usage and GC content from those of genes on the other chromosomes (13, 22). Second, a supernumerary chromosome in another plant-pathogenic fungus, *Colletotrichum gloeosporioides*, can be transferred laterally (14). Third, CD chromosomes are present in some, but not all, isolates of *N. haematococca*, and this DNA is not found in other portions of the genome (3). Finally, the *PEP* genes have also been shown to have a discontinuous phylogenetic distribution (42), another feature of horizontally transferred DNA. Since it has been demonstrated repeatedly that the rhizosphere is conducive to horizontal gene transfer between bacteria (7), we hypothesize that the *PDA1*-CD chromosome could have been obtained through horizontal gene transfer in the rhizosphere and maintained by environmental selection acting on clustered host-specifying genes.

The ability to use HS in fungi other than pea pathogens is rare; however, some fungi which are pathogenic on other legumes (e.g., *F. solani* f. sp. *phaseoli*) could also grow on this amino acid (35). HS is also present in the root exudates of chickpea plants (20), and it is apparently present in the vegetative tissue of the jack bean (37). Therefore, the HUT^+ phenotype might be beneficial for pathogenicity on other plants.

It will be interesting to examine whether the initial site of infection by pea-pathogenic fungi on pea roots occurs at a site of HS release, the lateral roots in the pea plant (44). Since in pea plants HS has also been shown to accumulate in large amounts in the vegetative tissue because it is used for the storage and transport of carbon and nitrogen (31), it is also possible that HS utilization might be important in nutrient acquisition even after the fungus has infected the plant. Testing the effect of the *HUT* gene(s) on pathogenicity or rhizosphere competency will be possible once the genes for HS utilization are characterized, a feat apparently not yet accomplished for any microorganisms.

ACKNOWLEDGMENTS

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APPENDIX B: THE EFFECT OF CONDITIONALLY DISPENSABLE CHROMOSOMES OF *NECTRIA HAEMATOCOCCA* MATING POPULATION VI (MPVI) ON RHIZOSPHERE COLONIZATION OF SOYBEAN, TOMATO, DIPLOID ALFALFA AND PEA.

Abstract

The fungus *Nectria haematococca* MPVI is a habitat-diverse fungus and previous studies have shown that habitat-determining genes can be found on supernumerary chromosomes called "conditionally dispensable" (CD) chromosomes. In this study real-time PCR was used to determine if the *PDA1* CD chromosome or the *PDA6* CD chromosome confer a competitive advantage in the rhizosphere of the following plants: soybean (*Glycine max*), tomato (*Solanum lycopersicum*), and diploid alfalfa (*Medicago truncatula*). Previous studies have demonstrated that the *PDA1* CD chromosome gave this fungus a competitive advantage in the pea rhizosphere and in this study the *PDA6* CD chromosome was tested for this ability. The results presented here indicate that both the *PDA1* and *PDA6* CD chromosomes confer a competitive advantage in the rhizosphere of soybean, whereas only the *PDA6* CD chromosome confers a competitive advantage in the rhizospheres of tomato and alfalfa, and only the *PDA1* CD chromosome confers a competitive advantage in the rhizosphere of pea.

Introduction

For over a century it has been known that the number of microorganisms in the rhizosphere is higher than that in bulk soil (Hiltner, 1904). This "rhizosphere effect" is believed to be due to the growth of rhizosphere microorganisms that depend on soluble compounds released by plant roots as their major nutrient sources (Lynch and Whipps

1990). Therefore, the ability of bacteria and fungi to grow on compounds found in the rhizosphere is essential for their efficient colonization of the rhizosphere. In terms of plant health, and certainly in terms of quantities, fungi and bacteria are the two most important organisms found in the rhizosphere. However, very little is known about the nutrition of these groups of organisms in this complex ecological niche. On a quantitative basis, amino acids, sugars, and organic acids are the major components of root exudates, which are assumed to be the major sources of nutrients for microorganisms that colonize the rhizosphere.

As well as being used as sources of nutrients for rhizosphere microorganisms, root exudate compounds have also been shown to be chemo-attractants for various rhizosphere inhabitants. Organic acids and amino acids in soybean root exudates have been shown to induce a chemotactic response from *Bacillus megaterium*, a soybean root-colonizing bacterium and potential control agent (Zheng and Sinclair 1996). Barbour et al (1991) showed that both amino acids and organic acids in soybean root exudates were strong chemo-attractants for the nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum*. The chemotactic response to root and seed exudates may indeed be one of several important traits involved in the establishment of root colonization by bacteria (Weller 1988). Host plant flavonoids in root exudates have also been shown to induce a chemotactic response from the zoospores of *Aphanomyces euteiches* and *Phytophthora sojae*, pathogens of pea and soybean, respectively (Yokosawa et al. 1986, Morris and Ward 1992, Tyler et al. 1996).

Various compounds in root exudates have also been shown to be fungal and Oomycete spore germination inducers. El-Hamalawi and Erwin (1986) found that both amino acids and the sugar ribose in the root extracts and exudates of alfalfa, pea, and tomato plants stimulated oospore germination of *Phytophthora megasperma* f. sp. *medicaginis*. Morris and Ward (1992) showed that some soybean isoflavonoids act as specific chemotaxis agents for *Phytophthora sojae* zoospores. Of particular interest to the current study, Ruan et al. (1995) found that various specific host flavonoids of pea and bean stimulated macroconidia germination *of Fusarium solani* f. sp. *pisi* (host = pea) and f. sp. *phaseoli* (host = bean), respectively. Their results indicated that the germination response was highly specific for certain host plant flavonoids, not merely due to utilization of these flavonoids as nutritional sources (Ruan et al. 1995). This is an example of how a plant pathogen has adapted to recognize host plant chemicals, a necessary component of the response of a specialized pathogen to its host.

In Appendix A of this work it was shown that the fungus *Nectria haematococca* mating population (MP) VI, a root rot pathogen of pea, can utilize the amino acid homoserine (HS), which is found in high quantities in pea (*Pisum sativum*) root exudates, as a sole carbon and nitrogen source (Rodriguez et al. 2008). It was also shown that the gene(s) for this trait was on a conditionally dispensable (CD) chromosome. CD chromosomes are supernumerary chromosomes that are not needed for axenic growth but contain genes that allow growth in different habitats (Covert 1998). I showed that the CD chromosome containing the gene(s) conferring the HS-utilization phenotype also gave *N.*

haematococca MPVI an increased competitive advantage in the pea rhizosphere (Rodriguez et al. 2008).

N. haematococca MPVI contains several different CD chromosomes and these can be differentiated by which *PDA* gene they contain (VanEtten et al. 2001). *PDA* genes encode pisatin demethylase, which detoxifies the pea phytoalexin pisatin (Matthews and VanEtten 1983, Kistler and VanEtten 1984, Miao et al. 1991). In the current study I use real-time PCR to determine if the *PDA1* CD chromosome or the *PDA6* CD chromosome confer a competitive advantage in the rhizosphere of the following plants: soybean (*Glycine max*), tomato (*Solanum lycopersicum*), and diploid alfalfa (*Medicago truncatula*). Various studies have identified a wide range of amino acids, sugars, and organic acids that are released as root exudates in these plants (Rovira 1959, Vancura and Hovadik 1965, Barbour et al. 1991, Zheng and Sinclair 1996, Simons et al. 1997, Lugtenberg et al. 1999, Menosso et al. 2001, Timotiwu and Sakurai 2002, Kravchenko et al. 2003, Phillips et al. 2004, Kamilova et al. 2006, Lopez et al. 2008). In addition, the *PDA6* CD chromosome, which does not confer the ability to use HS, was tested for its ability to confer a rhizosphere competitive ability on *N. haematococca* MPVI on pea.

Materials and Methods

Fungal strains

N. haematococca MPVI isolates used for this study were obtained from the culture collection of Dr. Hans VanEtten and are listed in Table B.1. Stock cultures of *N. haematococca* MPVI were maintained as slant cultures on V-8 agar medium (Stephens 1974). Cultures were incubated in the dark at 24±1^{o}C.

Table B.1. *Nectria haematococca* isolates used in this study.

Isolate	Source	CD chromosome	*Actin* gene	*HUT* genes	CD Marker: *PDA6-1*	CD Marker: *PDA1*	CD Marker: *cDNA3*
230-30-6	Ascospore isolate	*PDA6*[1]	+	na	+	na	na
230-CL-3	Derivative of 230-30-6; lost the *PDA6* CD chromosome by exposure to benomyl[2]	-	+	na	-	na	na
77-13-4	Ascospore isolate	*PDA1*[3]	+	+	na	+	+
Tr78.2	Derivative of 77-13-4; lost most of the *PDA1* CD chromosome during transformation but retained a portion that contains the *HUT* genes[4]	Has a portion of the *PDA1* gene that was part of the knock-out vector used to make this transformant; has portion of the CD chromosome that contains the *HUT* gene(s)[4]	+	+	na	+	-
HT1	Derivative of 77-13-4; lost chromosomes 14 (the *PDA1* CD chromosome) and 17 by exposure to benomyl[4]	-	+	-	na	-	-
HT5	Derivative of 77-13-4; lost chromosome 14 (*PDA1* CD chromosome) by exposure to benomyl[4]	-	+	-	na	-	-

[1] Miao et al. 1991
[2] VanEtten et al. 1998
[3] Maloney and VanEtten 1994
[4] Wasmann and VanEtten 1996, Rodriguez et al. 2008
na = not applicable

Spore production and collection

N. haematococca MPVI spores were produced in Petri dishes containing solidified V-8 agar medium (Stephens 1974). CD^{+} [1] and CD^{-} isolates used in this study had been previously cultured and examined to ensure that they had similar growth rates and production of micro- and macroconidia. Cultures were incubated at 24±1^{o}C under constant illumination to encourage production of macroconidia. Generally, cultures grown under these conditions gave similar percentages (~80-100%) of macroconidia (data not shown). After 1-2 wks of growth, the spores were harvested, rinsed with sterile water, suspended in water, and counted using a hemacytometer. For the rhizosphere competence assays the number of viable spores in the inocula was confirmed by dilution plating the spore suspensions on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) and counting fungal colonies. For inoculation of rhizospheres, macroconidia were counted as five cells since the vast majority of macroconidia produced by the *N. haematococca* isolates used had five nuclei, whereas microconidia were counted as single cells. For the purpose of spore count confirmation via culturing on PDA, both micro- and macroconidia were counted as single spores.

Rhizosphere Competition Assays

The replacement series technique (deWit 1960), as described in Appendix A, was used to measure rhizosphere competition as a function of the relative abundance of two competing *N. haematococca* MPVI isolates in the rhizospheres of pea, diploid alfalfa, soybean, and tomato plants.

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[1] CD^{+} = containing the CD chromosome; CD^{-} = not containing the CD chromosome

Seeds of tomato (cv. Better Boy; Park Seed Co., Greenwood, SC), diploid alfalfa (cv. Jemalong A17; courtesy of Dr. Douglas Cook, University of California, Davis, CA), soybean (cv. Ripley; courtesy of Dr. Randall Nelson, USDA Soybean Germplasm Collection, Urbana, IL), and pea (cv. Little Marvel; Royal Seeds, Kansas City, MO) were surface-sterilized in 70% ethanol for 5 min., in a 2.5% sodium hypochlorite solution for 10 min., and then rinsed thoroughly with sterile distilled water. Surface-sterilized seeds were planted (one seed per box for tomato, soybean, and pea; six seeds per box for alfalfa) in Magenta™ boxes (GA7, Magenta Corp., Chicago, IL) containing 100 g of sterile potting mixture (four parts vermiculite to one part quartz sand wetted with one L of sterile distilled water per 10 L of mixture).

Germination of seeds, as indicated by the appearance of the radical, generally took place within 1-5 days. Following germination, plants were grown under a 12-hour light, 12-hour dark regime at 24±1^{o}C temperature. After a one-week (for soybean) or two-week (for tomato, diploid alfalfa, and pea) growth period, the potting mixture was inoculated with spores of a single isolate or a combination of two isolates of *N. haematococca* MPVI. The first combination of isolates consisted of either HT1 or HT5, which are derivatives of 77-13-4 selected for loss of the *PDA1* CD chromosome (VanEtten et al. 1998) and either 77-13-4, which contains the *PDA1* CD chromosome (Maloney and VanEtten 1994), or Tr78.2, a transformant that lacks a wild-type copy of *PDA1* but contains the hygromycin resistance gene (*hph*) flanked by 692bp 5' and 888bp 3' of the *PDA1* gene (Wasmann and VanEtten 1996). The portion of *PDA1* from the transformation knockout vector was used in this study as a marker for Tr78.2.

Transformant Tr78.2 also carries a portion of the *PDA1* CD chromosome that contains the *HUT* gene(s) (Rodriguez et al. 2008, Appendix A). The second combination of isolates consisted of 230-30-6, which contains the *PDA6* CD chromosome (Miao et al. 1991), and 230-CL-3, which is a derivative of 230-30-6 selected for the loss of the *PDA6* CD chromosome (VanEtten et al. 1998). The final spore suspension was prepared to give a constant density of 10^5 *N. haematococca* MPVI spores per gram of potting mixture but at varying ratios of the two isolates. The ratios were 0:100, 50:50, and 100:0 for diploid alfalfa with 77-13-4:HT1, pea with 230-30-6:230-CL-3, and both tomato studies; and 0:100, 25:75, 50:50, 75:25, and 100:0 for diploid alfalfa with 230-30-6:230-CL-3, pea with both Tr78.2:HT1 and Tr78.2:HT5, and both soybean studies. Some repeat experiments were performed using only the 100:0, 50:50, and 0:100 ratios. The plants were grown for another two weeks (for initial pea experiments), three weeks (for soybean, tomato, and later pea experiments) or four weeks (for diploid alfalfa experiments) under the same conditions. During the growth period, the plants were watered with Hoagland's solution every 4-6 days.

At the end of the 2-4 wk period, the rhizospheres and roots were harvested by cutting the stem immediately above the uppermost roots and gently shaking off the loose potting mixture. The harvested roots and adhering potting mixture were lyophilized and ground to a fine powder in liquid nitrogen using a mortar and pestle. DNA for real-time PCR was extracted from one gram of ground rhizosphere material using the Ultraclean™

Soil DNA Kit (Mo Bio Laboratories, Inc., Solana Beach CA). Three to six[1] replicates were performed for each ratio of *N. haematococca* MPVI isolates. All experiments were repeated at least once. Non-inoculated plants and no-plant inoculated soils served as controls for these experiments.

Real-time quantitative RT-PCR

The number of cells in each sample was determined from the target gene copy number, which was quantified by comparing the Ct value of the samples to the Ct value of the respective standard curve. Standard curves for fungal marker genes were constructed with serial dilutions of the PCR products obtained from the genomic DNA of either *N. haematococca* MPVI isolate 77-13-4, which has the *PDA1* CD chromosome, or *N. haematococca* MPVI isolate 230-30-6, which has the *PDA6-1* CD chromosome. Standard curves for the plant gene rubisco activase (*RCA*) were constructed with serial dilutions of the PCR products obtained from the genomic DNA of tomato, soybean, pea, or diploid alfalfa. PCR products for standard curves were produced by using the same primer pairs as those used for the real-time quantitative PCR of rhizosphere samples. The *N. haematococca* MPVI actin gene (Liu et al. 2003) was used for real-time PCR to determine the total number of *N. haematococca* MPVI cells in the rhizosphere samples. The *N. haematococca* MPVI *PDA1* gene (Maloney and VanEtten 1994), the *PDA6-1* gene (Miao et al. 1991), or the cDNA3 gene (Han et al. 2001), which are all on the *PDA1* CD chromosome, were used for real-time PCR to determine the number of CD^+ (i.e. 77-

[1] For soybean four replicates were used for experiments with 230-30-6:230-CL-3 and three replicates were used for experiments with 77-13-4:HT1; for pea, tomato, and diploid alfalfa at least six replicates were used for all experiments.

13-4 or 230-30-6) or *HUT*$^{+}$ (i.e. Tr78.2) *N. haematococca* MPVI cells in the rhizosphere samples. Real-time quantitative PCR using TaqMan® technology was performed on the Abbott Prism® System (Abbott Park, Illinois) according to the manufacturer's protocol. Sequences of the primers (Invitrogen Corporation, Carlsbad, California) and TaqMan® fluorescent probes (Applied Biosystems, Foster City, CA) used in the quantitative real-time PCR study are listed in Table B.2.

Each TaqMan® probe was designed to anneal to a specific sequence between the forward and reverse primers of its target gene and to have at least a 5^{o}C higher T_m than the PCR primers. Individual probes contain a reporter fluorochrome (6-carboxyfluorescein [6-FAM] for *PDA-1*, *PDA6-1*, cDNA3, and actin, tetrachloro-6-carboxyfluorescein [TET] for *RCA*) at the 5' end and a quencher fluorochrome (6-carboxy-tetramethyl-rhodamine [TAMRA]) at the 3' end. PCR analyses were performed in duplicate, with duplicate reactions on each run. Each reaction had a total volume of 25 µl containing 12.5 µl of qPCR® Mastermix Plus (Invitrogen Corp., Carlsbad, CA; contains reaction buffer dNTPs (including dUTP), Hot Goldstar® DNA polymerase, 5 mM $MgCl_2$, uracil-N-glycosylase, stabilizers, and passive reference), 300 nM forward primer, 300 nM reverse primer, 200 nM Taqman® probe, 2 µl rhizosphere DNA template, and 9 µl of sterile distilled water. The PCR parameters were as follows: an initial uracil-N-glycosylase step at 50^{o}C for 2 min, followed by an initial denaturation and Hot Goldstar® DNA polymerase activation step at 95^{o}C for 10 min., and 40 cycles at 95^{o}C for 15 sec. and 60^{o}C for 30 sec. Real-time PCR for each gene was performed at least three times.

Table B.2. Primers and probes used in this study.

***Nectria haematococca* primers and probes used for all rhizosphere experiments**	
Actin forward primer	5'-ATCCACG- TCACCACCTTCAA-3'
Actin reverse primer	5'-GTGCCAGAGTTAGAAATGATC-3'
Actin probe	FAM-ACATCGACATCACACTTCATGATGGAG-TAMRA
PDA1 forward primer	5'-GATGAGCAGACTGAGGTTGGT-3'
PDA1 reverse primer	5'-CTGTGATGCCAAGGTCACTTA-3'
PDA1 probe	FAM-TGCCTGCAGTGCCCTGGACG-TAMRA
PDA6-1 forward primer	5′-GGCGACGACGATGCACTC-3'
PDA6-1 reverse primer	5′-GCCTGCTGAGTCTCTGTCGAA-3'
PDA6-1 probe	FAM-CGAGAACAACTTCCTGGCCAAGCTGA-TAMRA
cDNA3 forward primer	5′-CGAACGAATCAACCTGTTAGT-3'
cDNA3 reverse primer	5′-GCATCAGAAGCAACCTGATC-3'
cDNA3 probe	FAM-TCG CGC CAT GCA CAA CAG CAA AC-TAMRA
Primers and probes used for Pea	
RCA forward primer	5'-CTTGTTCATCAACGATCTTGAT-3'
RCA reverse primer	5'-GGTTGTCAGCAATGTTCATGAG-3'
RCA probe	TET-CACCGTCAACAACCAGATGGTGAATG-TAMRA
Primers and probes used for Tomato	
RCA forward primer	5′-CCTCTTCATCAACGATCTCGAT-3'
RCA reverse primer	5′-GGTTGTCAGCAATGTTCATGAG-3'
RCA probe	TET-CACCGTCAACAACCAGATGGTGAATG-TAMRA
Primers and probes used for Soybean	
RCA forward primer	5'-CATTATGATGAGTGCTGGAGA-3'
RCA reverse primer	5′-GGTTGTCAGCAATGTTCATGAG-3'
RCA probe	TET-CACCGTCAACAACCAGATGGTGAATG-TAMRA
Primers and probes used for Diploid Alfalfa	
RCA forward primer	5′-CTTGTTCATCAACGATCTTGAT-3'
RCA reverse primer	5′-GGTTGTCAGCAATGTTCATGAG-3'
RCA probe	TET-CACCGTCAACAACCAGATGGTGAATG-TAMRA

deWit Replacement Curves

The *RCA*[1] gene from pea, tomato, soybean, and diploid alfalfa was used to measure the number of corresponding plant cells in samples and to normalize the amount and quality of the genomic DNA. *RCA* mRNA sequences were obtained from the GenBank sequence database for 14 plant species and aligned using the Clustal function of MacVector™ software. The region with the highest identity across species were used to design the PCR forward, 5'-CATTATGATGAGTGCTGGAGA-3' and reverse, 5'-TCCATACGACCATCACGGAT-3' primers. These primers were used to amplify the corresponding ~350-bp region in the *RCA* genes of pea, tomato, and diploid alfalfa using genomic DNA of pea cv. Little Marvel, tomato cv. Better Boy, and diploid alfalfa cv. Jemalong A17 as templates. The PCR amplification products obtained were then sequenced and those *RCA* DNA sequences were used to design the PCR primers for pea, tomato, and diploid alfalfa *RCA*. PCR primers for soybean *RCA* were designed using the soybean *RCA* cDNA sequence (Accession Number BI944454) obtained from the GenBank sequence database. Enough similarity was found between the pea, tomato, soybean, and diploid alfalfa *RCA* sequences to use the Taqman® probe that had been previously designed for pea (see Appendix A).

Competition between the CD^+ and CD^- isolates was shown diagrammatically by plotting the results on a deWit replacement curve, on which dashed lines represent the growth of the isolates without competition and solid lines represent the actual growth

[1] When this assay was designed, it was thought that all plants had a single copy of *RCA*. It is now known that some plants have two copies (Portis 2003). The *RCA* copy number of pea, tomato, soybean, and diploid alfalfa is unclear. Thus the values for the number of fungal cells per plant cell may be two-fold higher than reported in these figures.

under competition. If there is no competition between the two isolates, i.e., each can colonize the rhizosphere equally, then the ratios of the two isolates recovered from the rhizosphere of plants inoculated with CD^+ (or portion thereof) and CD^- mixtures should be the same as the inoculation ratios.

Analysis of substrate utilization by *N. haematococca* MPVI isolates

Biolog GP2 and GN2 Microplates™ (Biolog, Hayward, CA) were used in an attempt to identify substrates that can be differentially utilized as carbon and/or nitrogen sources by *N. haematococca* MPVI isolates with and without the CD chromosomes. The Biolog GP2 and GN2 Microplates™ were used according to manufacturer's instructions. Additional organic acids, sugars, and amino acids found in the root exudates or root extracts of the plant species used in this study were evaluated by using a modified M-100 medium (as described in Appendix A). The compounds tested and their concentrations were: citric acid (20g/L; Sigma-Aldrich, St. Louis, MO), succinic acid (20g/L; 3-O-methyl-D-chiro-inositol; Fisher Scientific, Fair Lawn, NJ), malic acid (20g/L; Nutritional Biochemicals Corp., Cleveland, OH), xylose (20g/L; Sigma-Aldrich, St. Louis, MO), pinitol (5g/L; Sigma-Aldrich, St. Louis, MO), D-glucuronic acid (10g/L; Sigma-Aldrich, St. Louis, MO), or D-galacturonic acid (20g/L; Sigma-Aldrich, St. Louis, MO) as sole carbon sources, or DL-isoleucine (5g/L MP Biomedicals Inc., Aurora, OH), L-isoleucine (5g/L; Sigma-Aldrich, St. Louis, MO), DL-lysine (5g/L; MP Biomedicals Inc., Aurora, OH), L-lysine (10g/L; Sigma-Aldrich, St. Louis, MO), phosphoserine (5g/L; Sigma-Aldrich, St. Louis, MO), allantoin (10g/L; Sigma-Aldrich, St. Louis, MO), or L-citrulline

(10g/L; Sigma-Aldrich, St. Louis, MO) as sole carbon and nitrogen sources. Approximately 1000-5000 spores of the different *N. haematococca* MPVI isolates were inoculated onto the semi-solid medium and the plates were incubated in the dark at 24±1^{o}C. Cultures were examined for growth after 5 days.

Results

Competitive ability of isolates with and without the CD chromosomes in the pea rhizosphere

As previously presented in Appendix A of this work, two wks after the soil mix containing the pea seedlings was inoculated with 25:75, 50:50, and 75:25 ratios of isolates Tr78.2:HT1, there was a significant change ($p < 0.05$) from the initial ratios of each isolate such that isolate Tr78.2, which contains part of the *PDA1* CD chromosome and can utilize homoserine as a sole carbon and nitrogen source, was 31%, 60%, and 90%, respectively, of the total fungal biomass in the rhizosphere, while isolate HT1, which contains no part of the *PDA1* CD chromosome and cannot use homoserine as a sole carbon and nitrogen source, was only 69%, 40%, and 10%, respectively, of the fungal biomass (Table B.3)[1] and (Figure 5 of Appendix A). The repeat experiment gave similar results with isolate Tr-78.2 accounting for 87% of the fungal biomass two wks after inoculation with a 50:50 mixtures of both isolates (data not shown). Since isolate HT1 is actually missing two CD chromosomes (chromosomes 14, the *PDA1* CD chromosome, as well as chromosome 17), an additional CD^{-} isolate, HT5, which is

[1] Reiterated here for the purpose of comparison with the experiments using other plant species.

Table B.3. DNA was extracted from pea rhizospheres 2 weeks post-inoculation with *N. haematococca* MPVI isolates Tr78.2 and HT1 and the biomass of each isolate was determined using real-time PCR. The means are significantly different from expected results for the 25:75, 50:50, and 75:25 treatments (Newman-Keuls Multiple Comparison Test; $p < 0.05$).

Initial inoculum	**3-weeks post-inoculation**
***PDA1*$^{+}$: *PDA1*$^{-}$** [1]	***PDA1* as % of *actin* (Mean ± SE)**
0:100	0 ± 0
25:75	30 ± 4
50:50	61 ± 6
75:25	90 ± 3
100:0	99 ± 5

[1] Portion of *PDA1* gene that was a part of the knock-out vector used to make Tr78.2 was used as marker**.**

missing only chromosome 14 (Appendix A; Dr. M. Taga, personal comm.), was used with Tr78.2 to determine that the decreased rhizosphere competitive ability of HT1 was not due to the absence of chromosome 17. Three wks after the soil mix containing the pea seedlings was inoculated with 25:75, 50:50, and 75:25 ratios of isolates Tr78.2:HT5, there was a significant change ($p < 0.05$) from the initial ratios of each isolate such that isolate Tr78.2 was 42%, 71%, and 93%, respectively, of the total fungal biomass in the rhizosphere, while isolate HT1 was only 58%, 29%, and 7%, respectively, of the fungal biomass (Table B.4 and Figure B.1). That experiment was repeated with similar results. Thus, the reason for the decrease in competitive ability of both isolates HT1 and HT5 would appear to be due to the absence of the portion of the chromosome 14 that contains the *HUT* gene(s). In these studies using isolates Tr78.2, HT1, and HT5 the inoculated roots did not show any disease symptoms, although occasionally very small regions of necrosis were seen on the pea epicotyls. No disease symptoms were observed with any other combination of *N. haematococca* MPVI isolates and plants. In these experiments with Tr78.2, HT1, and HT5, and all subsequent experiments detailed below, no *N. haematococca* MPVI was detected in the rhizosphere of non-inoculated controls and no significant increase of *N. haematococca* MPVI biomass was detected in no-plant inoculated soils.

Two wks after the pea rhizosphere was inoculated with 25:75, 50:50, and 75:25 ratios of isolates 230-30-6:230-CL-3, there was no significant change from the initial ratios of each isolate. For example, in the 50:50 ratio the *PDA6* CD^+ isolate 230-30-6 was

Table B.4. DNA was extracted from pea rhizospheres 3 weeks post-inoculation with *N. haematococca* MPVI isolates Tr78.2 and HT5 and the biomass of each isolate was determined using real-time PCR. The means are significantly different from expected results for the 25:75, 50:50, and 75:25 treatments (Newman-Keuls Multiple Comparison Test; $p < 0.05$).

Initial inoculum ***PDA1*$^+$: *PDA1*$^-$** [1]	**3-weeks post-inoculation** ***PDA1* as % of *actin* (Mean ± SE)**
0:100	1 ± 0[2]
25:75	42 ± 2
50:50	71 ± 5
75:25	93 ± 2
100:0	100 ± 1

[1] Portion of *PDA1* gene that was a part of the knock-out vector used to make Tr78.2 was used as marker**.**

[2] An occasional false positive result has been observed with these studies. As these were always very small values and observed even with non-inoculated plants, they were interpreted as reflection of background noise in the assay.

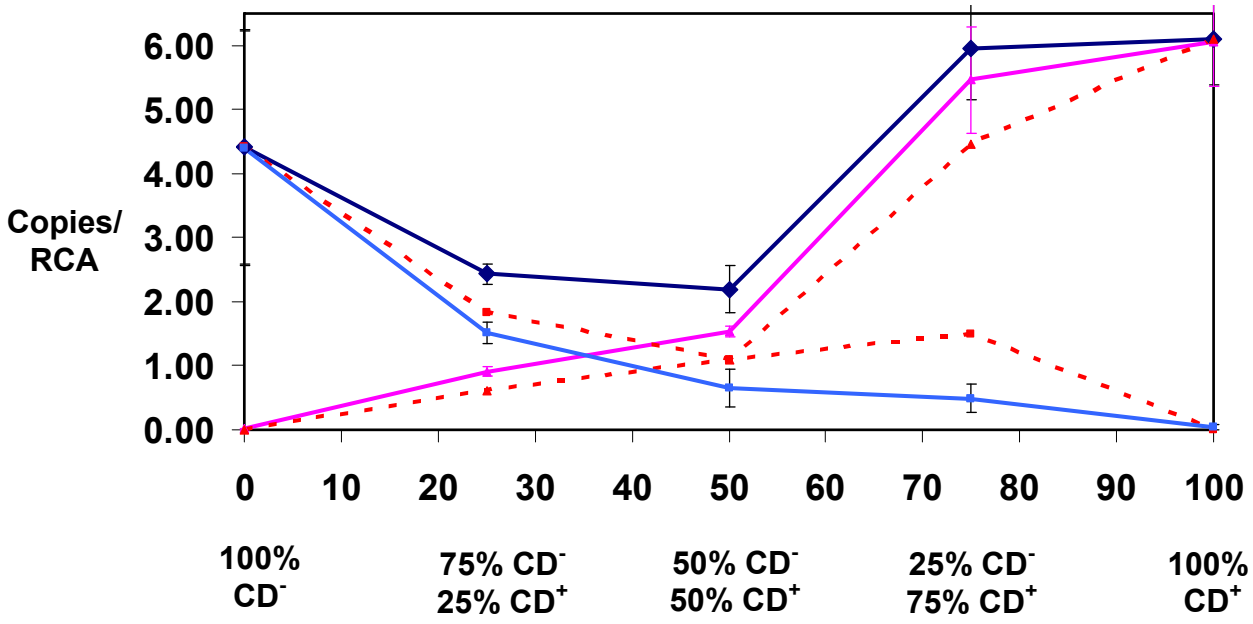

Figure B.1. The deWit Replacement Diagram of data for pea shown in Table B.4. There is better pea rhizosphere colonization by the isolate containing a portion of the *PDA1* CD chromosome (Tr78.2; pink line) than by the isolate without that portion of the CD chromosome (HT5, blue line), as indicated by a higher-than-expected ratio of *PDA1* relative to *actin* gene. Tr78.2 accounts for 42%, 71%, and 93% of the total fungal biomass 3-weeks post-inoculation with 25:75, 50:50, and 75:25 mixtures. The mean is significantly different from the expected result for the 50:50 treatment (Newman-Keuls Multiple Comparison Test; $p < 0.05$). Six replicates were performed for each treatment.

47% of the total fungal biomass, while the *PDA6* CD^- isolate 230-CL-3 was 53% of the fungal biomass (data not shown).

Competitive ability of isolates with and without the CD chromosomes in the tomato rhizosphere

Three wks after the soil mix containing tomato seedlings was inoculated with a 50:50 ratio of 230-30-6:230-CL-3, there was a significant increase ($p < 0.05$) in the relative ratio of 230-30-6 from the initial ratios of each isolate (Table B.5, Figure B.2). A repeat experiment gave similar results with isolate 230-30-6 accounting for 63% of the fungal biomass three weeks after inoculation with a 50:50 mixture of both isolates (data not shown). However, 3 wks after the tomato rhizosphere was inoculated with a 50:50 ratio of 77-13-4: HT1, there was no significant change from the initial ratios as the *PDA1* CD^+ isolate 77-13-4 was 53% of the total fungal biomass, while the *PDA1* CD^- isolate HT1 was 47% of the fungal biomass (data not shown). The experiment was repeated with similar results.

Competitive ability of isolates with and without the CD chromosomes in the soybean rhizosphere

Three wks after the soil mix containing the soybean seedlings was inoculated with 25:75, 50:50, and 75:25 ratios of isolates 230-30-6:230-CL-3, there was a significant increase ($p < 0.05$) in the relative ratio of 230-30-6 from the initial ratios of each isolate (Table B.6, Figure B.3). Similarly, 3 wks after the soil mix containing the soybean seedlings were inoculated with 25:75, 50:50, and 75:25 ratios of 77-13-4:HT1, there was

Table B.5. DNA was extracted from tomato rhizospheres 3 weeks post-inoculation with *N. haematococca MPVI* isolates 230-30-6 and 230-CL-3 and the biomass of each isolate was determined using real-time PCR. The mean is significantly different from the expected result for the 50:50 treatment (Newman-Keuls Multiple Comparison Test; $p < 0.05$).

Initial inoculum CD^+**:** CD^-	**3-weeks post-inoculation**
	***PDA6-1* as % of *actin* (Mean ± SE)**
0:100	0 ± 1
50:50	73 ± 2
100:0	100 ± 2

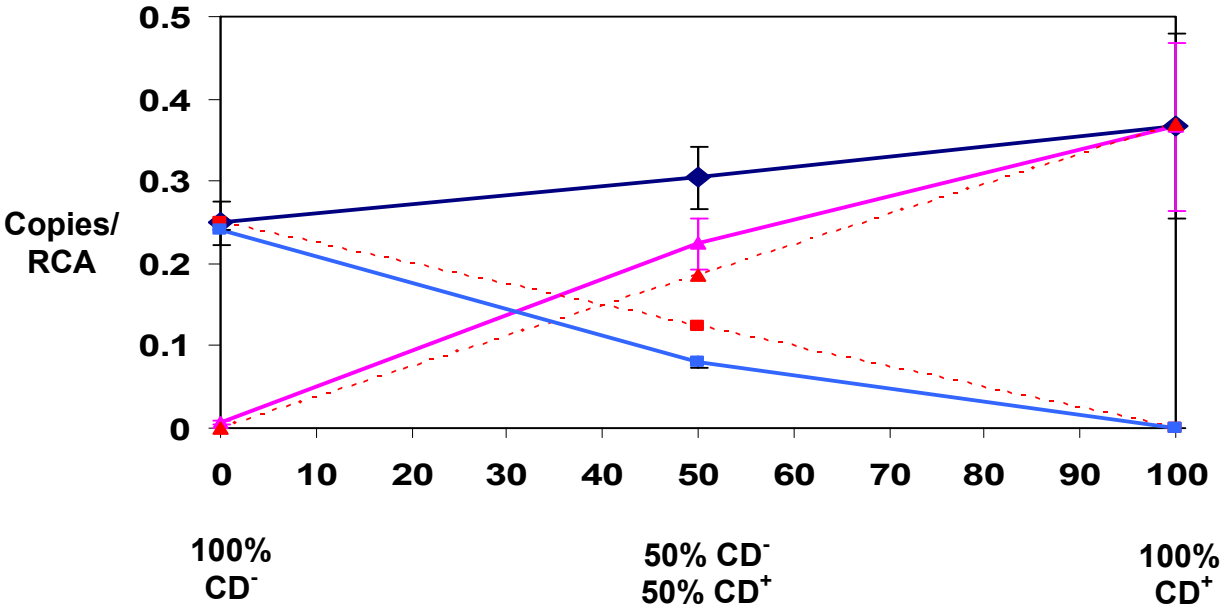

Figure B.2. The deWit Replacement Diagram of data for tomato shown in Table B.5. There is better tomato rhizosphere colonization by the isolate with the CD chromosome (230-30-6, pink line) than by the isolate without the CD chromosome (230-CL-3, blue line), as indicated by a higher *actin* copy number in samples inoculated with single isolates. In 50:50 mixtures of the CD^+ and CD^- isolates, competition in favor of the CD^+ isolate is indicated by a higher-than-expected ratio of *PDA6-1* relative to *actin* gene. The CD^+ isolate accounts for 73% of the total fungal biomass 3-weeks post-inoculation with a 50:50 mixture. The mean is significantly different from the expected result for the 50:50 treatment (Newman-Keuls Multiple Comparison Test; $p < 0.05$). Six replicates were performed for each treatment.

Table B.6. DNA was extracted from soybean rhizospheres 3 weeks post-inoculation with *N. haematococca* MPVI isolates 230-30-6 and 230-CL-3 and the biomass of each isolate was determined using real-time PCR. The means are significantly different from expected results for the 25:75, 50:50, and 75:25 treatments (Newman-Keuls Multiple Comparison Test; $p < 0.05$).

Initial inoculum CD^+**:** CD^-	**3-weeks post-inoculation**
	***PDA6-1* as % of *actin* (Mean ± SE)**
0:100	2 ± 0
25:75	63 ± 7
50:50	79 ± 4
75:25	86 ± 5
100:0	99 ± 5

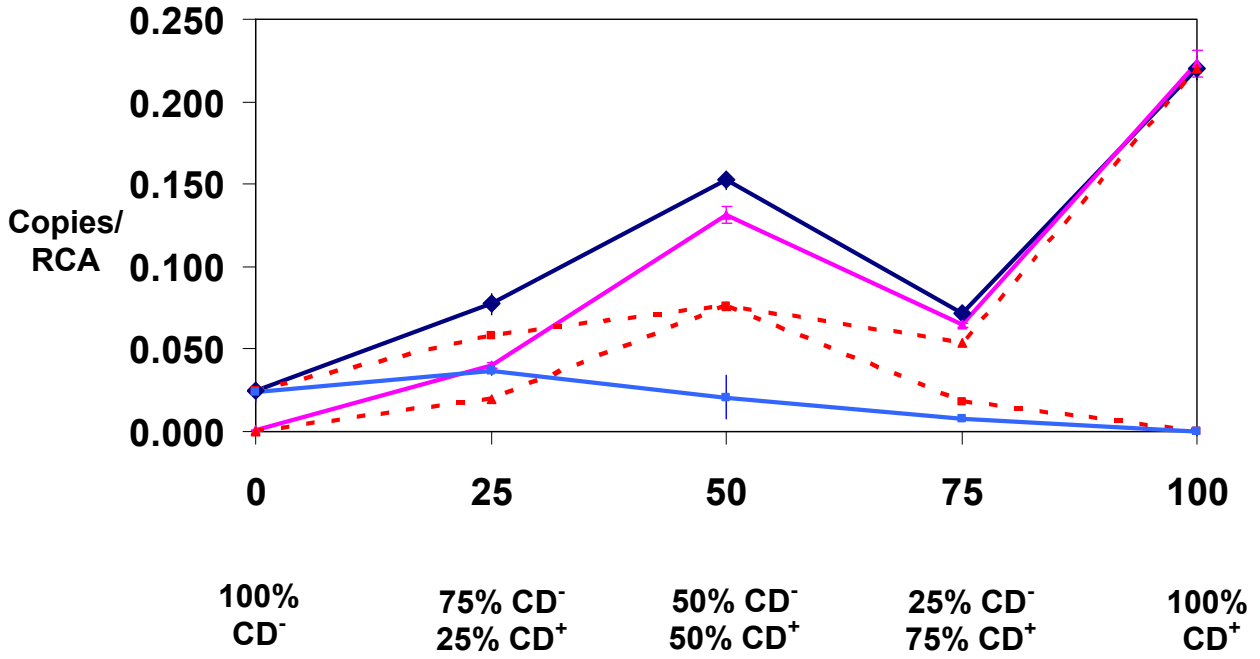

Figure B.3. The deWit Replacement Diagram of data for soybean shown in Table B.6. There is better soybean rhizosphere colonization by the isolate with the CD chromosome (230-30-6, pink line) than by the isolate without the CD chromosome (230-CL-3, blue line), as indicated by a higher *actin* copy number in samples inoculated with single isolates. In mixtures of the CD^+ and CD^- isolates, competition in favor of the CD^+ isolate is indicated by higher-than-expected ratios of *PDA6* relative to the *actin* gene. The CD^+ isolate accounts for 63%, 79%, and 86% of the total fungal biomass 3-weeks post-inoculation with 25:75, 50:50, and 75:25 mixtures. The mean is significantly different from the expected result for the 50:50 treatment (Newman-Keuls Multiple Comparison Test; $p < 0.05$). Four replicates were performed for each treatment.

a significant increase ($p < 0.05$) of the *PDA1* CD^+ isolate 77-13-4 from the initial ratios of each isolate (Table B.7 and Figure B.4). These experiments were repeated with similar results.

Competitive ability of isolates with and without the CD chromosomes in the diploid alfalfa rhizosphere

Four wks after the soil mix containing the diploid alfalfa seedlings was inoculated with a 50:50 ratio of isolates 230-30-6:230-CL-3, there was a significant increase ($p < 0.05$) in the relative ratio of the *PDA6* CD^+ isolate 230-30-6 from the initial ratio of each isolate as it was 87% of the total fungal biomass in the rhizosphere, while the *PDA6* CD^- isolate 230-CL-3 was only 13% (Table B.8, Figure B.5). A repeat experiment using 25:75, 50:50, and 75:25 ratios gave results showing a significant change from the initial ratios of each isolate such that isolate 230-30-6 was 30%, 66%, and 81% of the fungal biomass 4 wks after inoculation (Table B.9). However, 4 wks after the diploid alfalfa rhizosphere was inoculated with a 50:50 ratio of 77-13-4:HT1, there was no significant change from the initial ratios of each isolate. The *PDA1* CD^+ isolate 77-13-4 was 46% of the total fungal biomass, while the *PDA1* CD^- isolate 230-CL-3 was 54% of the fungal biomass (data not shown).

Table B.7. DNA was extracted from soybean rhizospheres 3 weeks post-inoculation with *N. haematococca* MPVI isolates 77-13-4 and HT1 and the biomass of each isolate was determined using real-time PCR. The means are significantly different from expected results for the 25:75, 50:50, and 75:25 treatments (Newman-Keuls Multiple Comparison Test; $p < 0.05$).

Initial inoculum CD^+**:** CD^-	**3-weeks post-inoculation**
	***PDA1* as % of *actin* (Mean ± SE)**
0:100	3 ± 1
25:75	39 ± 5
50:50	74 ± 6
75:25	88 ± 5
100:0	100 ± 6

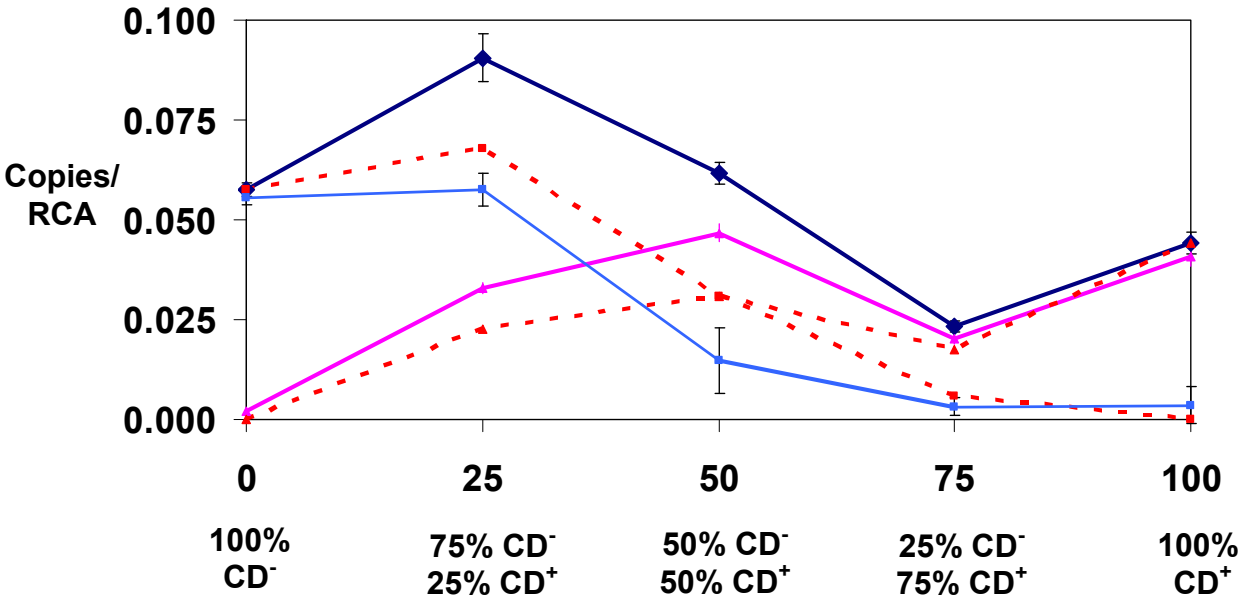

Figure B.4. The deWit Replacement Diagram of data for soybean shown in Table B.7. There is better soybean rhizosphere colonization by the isolate with the CD chromosome (77-13-4; pink line) than by the isolate without the CD chromosome (HT1; blue line), as indicated by a higher *actin* copy number in samples inoculated with single isolates. In mixtures of the CD^+ and CD^- isolates, competition in favor of the CD^+ isolate is indicated by higher-than-expected ratios of *PDA1* relative to the *actin* gene. The CD^+ isolate accounts for 39%, 74%, and 88% of the total fungal biomass 3-weeks post-inoculation with 25:75, 50:50, and 75:25 mixtures. The mean is significantly different from the expected result for the 50:50 treatment (Newman-Keuls Multiple Comparison Test; $p < 0.05$). Three replicates were performed for each treatment.

Table B.8. DNA was extracted from diploid alfalfa rhizospheres 4 weeks post-inoculation with *N. haematococca* MPVI isolates 230-30-6 and 230-CL-3 and the biomass of each isolate was determined using real-time PCR. The means are significantly different from expected results for the 50:50 treatment (Newman-Keuls Multiple Comparison Test; $p < 0.05$).

Initial inoculum CD^+**:** CD^-	**4 weeks post-inoculation**
	***PDA6-1* as % of *actin* (Mean ± SE)**
0:100	<1 ± 0
50:50	87 ± 4
100:0	98 ± 4

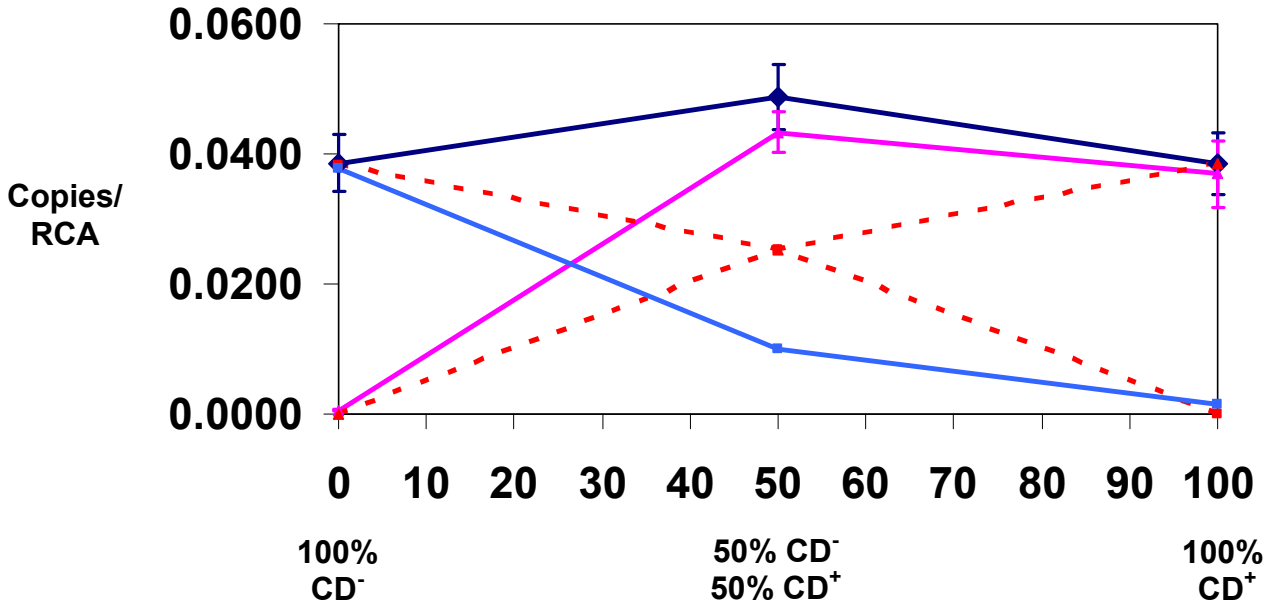

Figure B.5. The deWit Replacement Diagram of data for diploid alfalfa shown in Table B.8. In 50:50 mixtures of the CD^+ (230-30-6) and CD^- (230-CL-3) isolates, competition in favor of the CD^+ isolate is indicated by a higher-than-expected ratio of *PDA6* relative to *actin* gene. The CD^+ isolate accounts for 87% of the total fungal biomass 3-weeks post-inoculation with a 50:50 mixture. The mean is significantly different from the expected result for the 50:50 treatment (Newman-Keuls Multiple Comparison Test; $p < 0.05$). Six replicates were performed for each treatment.

Table B.9. DNA was extracted from diploid alfalfa rhizospheres 4 weeks post-inoculation with *N. haematococca* MPVI isolates 230-30-6 and 230-CL-3 and the biomass of each isolate was determined using real-time PCR. The means are significantly different from expected results for the 25:75, 50:50, and 75:25 treatments (Newman-Keuls Multiple Comparison Test; $p < 0.05$).

Initial inoculum CD^+**:** CD^-	**4 weeks post-inoculation**
	***PDA6-1* as % of *actin* (Mean ± SE)**
0:100	2 ± 1
25:75	30 ± 1
50:50	66 ± 2
75:25	81 ± 5
100:0	100 ± 5

Analysis of substrate utilization by CD chromosome$^+$ and CD chromosome$^-$ *N. haematococca* MPVI isolates

N. haematococca MPVI isolates 77-13-4, HT1, and HT5 gave the same results when inoculated into Biolog GP2 and GN2 plates, indicating that these isolates were capable of using the same nutrients present in those plates (Supplemental Table B.1 and B.2). Similarly, *N. haematococca* MPVI isolates 230-30-6 and 230-CL-3 gave the same results when inoculated into Biolog GP2 and GN2 plates, indicating that both of these isolates were capable of using the same nutrients present in those plates (Supplemental Table B.1 and B.2). Based on the identification of major organic acids, sugars, and amino acids found in the root exudates or root extracts of the plant species used in this study (Supplemental Tables B.3, B.4, and B.5), an attempt was made to test other compounds, which are not used in Biolog Microplates™, as carbon and/or nitrogen sources. *N. haematococca* MPVI isolates 77-13-4, HT1, and HT5 gave the same results when inoculated onto agar media containing these nutrients as did isolates 230-30-6 and 230-CL-3 (Supplemental Table B.6).

Discussion

Studies in our lab have showenthat some *N. haematococca* MPVI isolates can utilize HS as a sole carbon and nitrogen source and that the chromosome containing the gene(s) for that trait gave this fungus a competitive advantage in the rhizosphere compared to isolates of the fungus that lacked the gene(s) for that trait (Appendix A; Rodriguez et al. 2008). The observation that the *PDA6* CD chromosome, which does not confer a HUT$^+$ phenotype, does not give *N. haematococca* MPVI isolates a competitive

advantage on pea is also consistent with the hypothesis that it is the HUT gene(s) only that is responsible for the increased competitive advantage of *N. haematococca* MPVI with the *PDA1*-CD chromosome. Because these previous results indicated there were genes for rhizosphere competitiveness on one of the CD chromosomes, it is of interest to determine if there are other genes on the *PDA1* or *PDA6* CD chromosomes that give *N. haematococca* MPVI isolates having these chromosomes a similar competitive advantage in the rhizospheres of other plant species. Based on the ratios of the recovered biomass of the CD^+ versus the CD^- isolates from the rhizosphere, the current results suggest that both the *PDA6* and *PDA1* CD chromosomes give a competitive advantage of *N. haematococca* MPVI in the rhizosphere of soybean (Tables B.6 and B.7, Figures B.3 and B.4). In addition, the *PDA6* CD chromosome gives *N. haematococca* MPVI a competitive advantage in the rhizospheres of alfalfa (Tables B.8 and B.9, Figure B.5) and tomato (Table B.5, Figure B.2) but the *PDA1* CD chromosome does not.

In the previous analysis of possible competitive genes on the *PDA1* CD chromosome for colonizing the rhizosphere of pea the use of a plant gene as a common reference point for deWit curves indicated that the total fungal biomass, based on actin gene per RCA gene, was the same at the end of the experiment in all treatments (Appendix A, Fig 5). However, different values of biomass per plant cell (i.e., actin gene/RCA gene) were observed with different treatments (i.e. different ratios of spores used) in the repeat of the pea experiments with a different isolate (i.e., HT5 instead of HT1; Fig B.1). The same phenomenon was observed in experiments with other plants (Fig B.3 and B.4) and in repeat experiments in that the same total biomass per RCA was

not obtained in each treatment within the same experiment (Fig B.1 vs. Supplemental Fig. B.6). Since the direction of the relative changes (or lack of changes) in the final ratios of the isolates from the starting inoculum was consistent no matter what the starting ratio, it would appear that different amounts of plant tissue were being extracted in the different treatments. For example, a repeat of the experiment using pea inoculated with isolates Tr78.2:HT5 gave competition assay results similar to the original experiment (i.e., the original experiment results were 42%, 71%, and 93%, and the repeat experiment results were 38%, 76%, and 89%, for the 25:75, 50:50, and 75:25 ratios, respectively), but the ratios of fungal biomass per plant biomass were as much as three-fold higher for some of the treatments within the repeat experiment results (Fig. B.6). Therefore, it appears that this variation in the ratio of fungal biomass to RCA is the result of a technical issue and not the result of a biological phenomenon. The averages of the actin copy number to the *RCA* copy number for these experiments are still valuable representations of relative fungal to plant biomass and they still allow an indication of whether the differences are due to competition when there are differences. Examples of increased competitive ability by one isolate over another are demonstrated in this study where there is a higher yield of the *PDA6* CD^+ isolate and a lower yield of the *PDA6* CD^- isolate when tomato (Figure B.2), soybean (Figure B.3), and alfalfa (Figure B.5) are inoculated with mixtures of CD^+ and CD^- isolates. In these examples the deWit replacement curves show a higher than expected yield of the CD^+ isolate at the expense of the CD^- isolate. A similar increased competitive ability is demonstrated by the *PDA1* CD^+ isolate over the *PDA1* CD^- isolate in the rhizospheres of pea (Figure B.1) and soybean (Figure B.4). Also, despite the

inconsistencies in total fungal biomass per RCA gene in individual extractions, it is still obvious that some *N. haematococca* MPVI isolates are better colonizers of some plants than others. For example, when pea is inoculated with different ratios of Tr78.2:HT5, the average actin to RCA is 4.22 (Fig. B.1), but when soybean is inoculated with different ratios of 77-13-4:HT1, the average actin to RCA is 0.06 (Fig. B.4). Similarly, *N. haematococca* MPVI isolate 230-30-6 is a better colonizer of the tomato rhizosphere (Fig. B.2, average = 0.30) than of the soybean rhizosphere (Fig. B.3, average = 0.11).

As previously discussed, the power and versatility of real-time PCR is reflected by the use of this technique in various areas of scientific research, including medicine, environmental science, food microbiology, and plant pathology. Real-time PCR has also been previously used to quantify various fungal and Oomycete species in soil samples including *Helminthosporium solani* (Cullen et al. 2001), *Colletotrichum coccodes* (Cullen et al. 2002), *Rhizoctonia solani* (Lees et al. 2002), *Fusarium solani* f. sp. *phaseoli* (Filion et al. 2003a, 2003b), *Pythium oligandrum* (Takenaka et al. 2007), and *Phytophthora capsici* (Pavón et al. 2008). However, we believe that this study is the first use of real-time PCR to measure the competitive ability of different fungal isolates in the rhizosphere of plants. Various approaches for enumerating fungal biomass in soil, as reviewed in the dissertation introduction, have attempted to account for the fact that fungal propagules can represent either actual fungal biomass (i.e. hyphal growth) or merely spore production. The use of real-time PCR appears to overcome some of the previous difficulties, although this technique proved to be a somewhat laborious process and more expensive than traditional techniques for enumerating fungi in soil samples.

However, it did allow for a more precise quantification of fungal biomass as well as a method of estimating the actual fungal biomass relative to the plant biomass in rhizosphere samples. In addition, naturally-occurring gene differences between isolates, such as the *N. haematococca* MPVI *PDA1*, *PDA6*, and cDNA3 genes, could be used as markers for the real-time PCR, thus avoiding the need for prior genetic manipulations such as transformation to introduce marker genes.

Based on the finding that the *HUT* gene(s) appear to be basis of the competitive advantage of *N. haematococca* MPVI in the pea rhizosphere, I attempted to identify other root exudate compounds that could be used as nutrient sources differentially by CD^+ and CD^- *N. haematococca* MPVI isolates. Previous studies have identified a wide array of potential nutrient sources in root exudates of soybean, *M. sativa* (tetraploid alfalfa), *M. truncatula* (diploid alfalfa), and tomato (Supplemental Tables B.3, B.4, and B.5). Inoculation of a wide variety of potential nutrient sources (Supplemental Tables B.1, B.2, and B.6), including known root exudates, with the CD^+ and CD^- isolates used in this study showed that there were no differences that could be attributed to different abilities to use these compounds as carbon or nitrogen sources. The observed competitive advantage possessed by CD^+ isolates may possibly be due to other factors including components in the rhizosphere that preferentially induce spore germination or chemotaxis of CD^+ isolates.

The current studies show that both the *PDA6* and *PDA1* CD chromosomes give a competitive advantage of *N. haematococca* MPVI in the rhizosphere of several different plant species. Thus, they suggest the presence of genes on the *PDA6* and *PDA1* CD

chromosome that enhances the ability of *N. haematococca* to expand its habitat and support the idea, as discussed in Appendix A, that fungal CD chromosomes are analogous to host-specifying plasmids in plant-associated bacteria. The location of the rhizosphere competitive genes to a specific portion of the genome should help in identification of these genes. This is particularly true of the genes on the *PDA1* CD chromosome as the genome of *N. haematococca* MPVI isolate 77-13-4, which contains the *PDA1* CD chromosome, has been sequenced. The identification of the genes responsible for the competitive advantage of these CD^+ isolates could be accomplished using a several approaches. Further localization of the region of the *PDA1*-CD chromosome that contains rhizosphere competition genes could be accomplished by making a series of truncations in the CD chromosome and testing the effect of truncation on rhizosphere competency. An alternative approach, which was used for identification of the HUT genes (appendix C), is to test for rhizosphere competition genes by transforming each of the cosmids that span the CD chromosomes into a CD^- isolate and testing the transformants for rhizosphere competency.

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Supplemental Table B.1. Results of *N. haematococca* MPVI isolates substrate analysis using the Biolog GP2 Microplate™.

Biolog™ GP2 Nutrients	***N. haematococca* MPVI isolate**				
	77-13-4	**HT5**	**HT1**	**230-30-6**	**230-CL-3**
α-Cyclodextrin	-	-	-	-	-
β-Cyclodextrin	+	+	+	+	+
Dextrin	+	+	+	+	+
Glycogen	+	+	+	+	+
Inulin	+	+	+	+	+
Mannan	w	w	w	-	-
N-Acetyl-D-Glucosamine	+	+	+	+	+
N-Acetyl-β-D-Mannosamine	+	+	+	-	-
Amygdalin	+	+	+	+	+
L-Arabinose	+	+	+	+	+
D-Arabitol	+	+	+	+	+
Arbutin	+	+	+	+	+
D-Cellobiose	+	+	+	+	+
D-Fructose	+	+	+	+	+
L-Fucose	+	+	+	-	-
D-Galactose	+	+	+	+	+
D-Galacturonic acid	+	+	+	+	+
Gentiobiose	+	+	+	+	+
D-Gluconic acid	-	-	-	-	-
α-D-Glucose	+	+	+	+	+
m-Inositol	+	+	+	+	+
α-D-Lactose	+	+	+	w	w
Lactulose	+	+	+	-	-
Maltose	+	+	+	+	+
Maltotriose	+	+	+	+	+
D-Mannitol	+	+	+	+	+
D-Mannose	+	+	+	+	+
D-Melezitose	+	+	+	+	+
D-Melibiose	+	+	+	+	+
α-Methyl-D-Galactoside	+	+	+	+	+
β-Methyl-D-Galactoside	+	+	+	-	-
3-Methyl-Glucose	-	-	-	-	-
α-Methyl-D-Glucoside	+	+	+	+	+
β-Methyl-D-Glucoside	+	+	+	+	+
α-Methyl-D-Mannoside	+	+	+	-	-
Palatinose	+	+	+	+	+
D-Psicose	+	+	+	-	-
D-Raffinose	+	+	+	+	+

Supplemental Table B.1 (con't). Results of *N. haematococca* MPVI isolates substrate analysis using the Biolog GP2 Microplate™.

Biolog™ GP2 Nutrients	***N. haematococca* MPVI isolate**				
	77-13-4	**HT5**	**HT1**	**230-30-6**	**230-CL-3**
L-Rhamnose	+	+	+	+	+
D-Ribose	+	+	+	+	+
Salicin	+	+	+	+	+
Sedoheptulosan	w	w	w	-	-
D-Sorbitol	+	+	+	+	+
Stachyose	+	+	+	+	+
Sucrose	+	+	+	+	+
D-Tagatose	w	w	w	-	-
D-Trehalose	+	+	+	+	+
Turanose	+	+	+	+	+
Xylitol	+	+	+	-	-
D-Xylose	+	+	+	+	+
Acetic Acid	+	+	+	+	+
α-Hydroxybutyric Acid	w	w	w	-	-
β-Hydroxybutyric Acid	w	w	w	+	+
γ-Hydroxybutyric Acid	w	w	w	-	-
p-Hydroxy-Phenylacetic Acid	+	+	+	+	+
α-Ketoglutaric Acid	w	w	w	-	-
α-Ketovaleric Acid	+	+	+	w	w
Lactamide	+	+	+	-	-
D-Lactic Acid Methyl Ester	+	+	+	-	-
L-Lactic Acid	+	+	+	+	+
D-Malic Acid	+	+	+	+	+
L-Malic Acid	+	+	+	+	+
Pyruvatic AcidMethyl Ester	+	+	+	+	+
Succinic Acid Mono-methyl Ester	+	+	+	+	+
PropionicAcid	+	+	+	+	+
PyruvicAcid	+	+	+	+	+
SuccinamicAcid	+	+	+	+	+
SuccinicAcid	+	+	+	+	+
N-Acetyl-L-Glutamic Acid	w	w	w	-	-
L-Alaninamide	-	-	-	-	-
D-Alanine	+	+	+	+	+
L-Alanine	+	+	+	+	+
L-Alanyl-Glycine	+	+	+	+	+
L-Asparagine	+	+	+	+	+
L-Glutamic Acid	+	+	+	+	+
Glycyl- L-Glutamic Acid	+	+	+	-	-

Supplemental Table B.1 (con't). Results of *N. haematococca* MPVI isolates substrate analysis using the Biolog GP2 Microplate™.

Biolog™ GP2 Nutrients	***N. haematococca* MPVI isolate**				
	77-13-4	**HT5**	**HT1**	**230-30-6**	**230-CL-3**
L-Pyroglutamic Acid	+	+	+	+	+
L-Serine	+	+	+	+	+
Putrescine	+	+	+	w	w
2,3-Butanediol	w	w	w	-	-
Glycerol	+	+	w	w	w
Adenosine	-	-	-	-	-
2'-DeoxyAdenosine	-	-	-	-	-
Inosine	-	-	-	-	-
Thymidine	-	-	-	-	-
Uridine	-	-	-	-	-
Adenosine-5'-Monophosphate	-	-	-	-	-
Thymidine-5'-Monophosphate	-	-	-	-	-
Uridine-5'-Monophosphate	-	-	-	-	-
D-Fructose-6-Phosphate	-	-	-	-	-
α-D-Glucose-1-Phosphate	-	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-	-
D-L-α-Glycerol Phosphate	-	-	-	-	-

w = weakly positive

Supplemental Table B.2. Results of *N. haematococca* MPVI isolates substrate analysis using the Biolog GN2 Microplate™.

Biolog™ GN2 Nutrients	***N. haematococca* MPVI isolate**				
	77-13-4	**HT1**	**HT5**	**230-30-6**	**230-CL-3**
α-Cyclodextrin	-	-	-	-	-
Dextrin	+	+	+	+	+
Glycogen	+	+	+	+	+
N-Acetyl-D-Galactosamine	-	-	-	-	-
N-Acetyl-D-Glucosamine	+	+	+	+	+
Adonitol	-	-	-	-	-
L-Arabinose	+	+	+	+	+
D-Arabitol	+	+	+	+	+
D-Cellobiose	+	+	+	+	+
Erythritol	+	+	+	w	w
D-Fructose	+	+	+	+	+
L-Fucose	+	+	+	w	w
D-Galactose	+	+	+	+	+
Gentiobiose	+	+	+	+	+
α-D-Glucose	+	+	+	+	+
m-Inositol	+	+	+	+	+
α-D-Lactose	+	+	+	w	w
Lactulose	w	w	w	-	-
Maltose	+	+	+	+	+
D-Mannitol	+	+	+	+	+
D-Mannose	+	+	+	+	+
D-Melibiose	+	+	+	+	+
β-Methyl- D-Glucoside	+	+	+	+	+
D-Psicose	w	w	w	-	-
D-Raffinose	+	+	+	+	+
L-Rhamnose	+	+	+	+	+
D-Sorbitol	+	+	+	+	+
Sucrose	+	+	+	+	+
D-Trehalose	+	+	+	+	+
Turanose	+	+	+	+	+
Xylitol	-	-	-	-	-
Pyruvic Acid	+	+	+	-	-
Methyl Ester	+	+	+	-	-
Succinic Acid Mono-Methyl-Ester	+	+	+	-	-
Acetic Acid	-	-	-	-	-
Cis-Aconitic Acid	-	-	-	-	-
Citric Acid	-	-	-	-	-
Formic Acid	-	-	-	-	-

Supplemental Table B.2 (con't). Results of *N. haematococca* MPVI isolates substrate analysis using the Biolog GN2 Microplate™.

Biolog™ GN2 Nutrients	***N. haematococca* MPVI isolate**				
	77-13-4	**HT1**	**HT5**	**230-30-6**	**230-CL-3**
D-Galactonic Acid Lactone	-	-	-	-	-
D-Galacturonic Acid	+	+	+	+	+
D-Gluconic Acid	-	-	-	-	-
D-Glucosaminic Acid	-	-	-	-	-
D-Glucuronic Acid	-	-	-	-	-
α-Hydroxybutyric Acid	+	+	+	w	w
β-Hydroxybutyric Acid	+	+	+	+	+
γ-Hydroxybutyric Acid	w	w	w	w	w
p-Hydroxyphenylacetic Acid	+	+	+	+	+
Itaconic Acid	-	-	-	-	-
α-Keto Butyric Acid	+	+	+	w	w
α-Keto Glutaric Acid	-	-	-	w	w
α-Keto Valeric Acid	+	+	+	w	w
D,L-Lactic Acid	+	+	+	w	w
Malonic Acid	+	+	+	w	w
Propionic Acid	+	+	+	w	w
Quinic Acid	+	+	+	w	w
D-Saccharic Acid	+	+	+	+	+
Sebacic Acid	w	w	w	+	+
Succinic Acid	w	w	w	w	w
Bromosuccinic Acid	-	-	-	-	-
Succinamic Acid	w	w	w	+	+
Glucuronamide	-	-	-	-	-
L-Alaninamide	-	-	-	-	-
D-Alanine	+	+	+	+	+
L-Alanine	+	+	+	+	+
L-Alanylglycine	+	+	+	+	+
L-Asparagine	+	+	+	+	+
L-Aspartic Acid	+	+	+	+	+
L-Glutamic Acid	+	+	+	+	+
Glycyl-L-Aspartic Acid	-	-	-	-	-
Glycyl-L-Glutamic Acid	-	-	-	-	-
L-Histidine	-	-	-	-	-
Hydroxy-L-Proline	+	+	+	+	+
L-Leucine	+	+	+	+	+
L-Ornithine	+	+	+	+	+
L-Phenylalanine	+	+	+	+	+
L-Proline	+	+	+	+	+

Supplemental Table B.2 (con't). Results of *N. haematococca* MPVI isolates substrate analysis using the Biolog GN2 Microplate™.

Biolog™ GN2 Nutrients	***N. haematococca* MPVI isolate**				
	77-13-4	**HT1**	**HT5**	**230-30-6**	**230-CL-3**
L-Pyroglutamic Acid	+	+	+	+	+
D-Serine	+	+	+	+	+
L-Serine	+	+	+	+	+
L-Threonine	w	w	w	+	+
D,L-Carnitine	-	-	-	-	-
γ-Amino Butyric Acid	+	+	+	+	+
Urocanic Acid	-	-	-	-	-
Inosine	-	-	-	-	-
Uridine	-	-	-	-	-
Thymidine	-	-	-	-	-
Phenyethylamine	+	+	+	+	+
Putrescine	+	+	+	+	+
2-Aminoethanol	+	+	+	+	+
2,3-Butanediol	-	-	-	-	-
Glycerol	+	+	+	+	+
D,L-α-Glycerol Phosphate	-	-	-	-	-
α-D-Glucose-1-Phosphate	-	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-	-

w = weakly positive

Supplemental Table B.3. Components of soybean root exudates as cited in the literature.

Soybean Cultivar(s)	Component	Plant age	Major components			Reference(s)
			Organic acids	Sugars	Amino acids	
FT-1, Veneza, IAC-13, Paraná	Root exudate	9 days	citric acid, lactic acid, succinic acid, oxalic acid, malic acid			Menosso et al. (2001)
	Root exudate	15 days		glucose, pinitol, uronic acid, galactose		Timotiwu and Sakurai (2002)
Essex	Root exudate	2 days			aspartic acid, glutamic acid, alanine, arginine, serine, histidine	Barbour et al. (1991)
Hack, Williams 82	Root exudate	7 days			Similar amounts of 19 different amino acids, including those from Barbour et al (1991) study	Zheng and Sinclair (1996)

Supplemental Table B.4. Components of *M. sativa*[1] and *M. truncatula* root extracts and root exudates as cited in the literature.

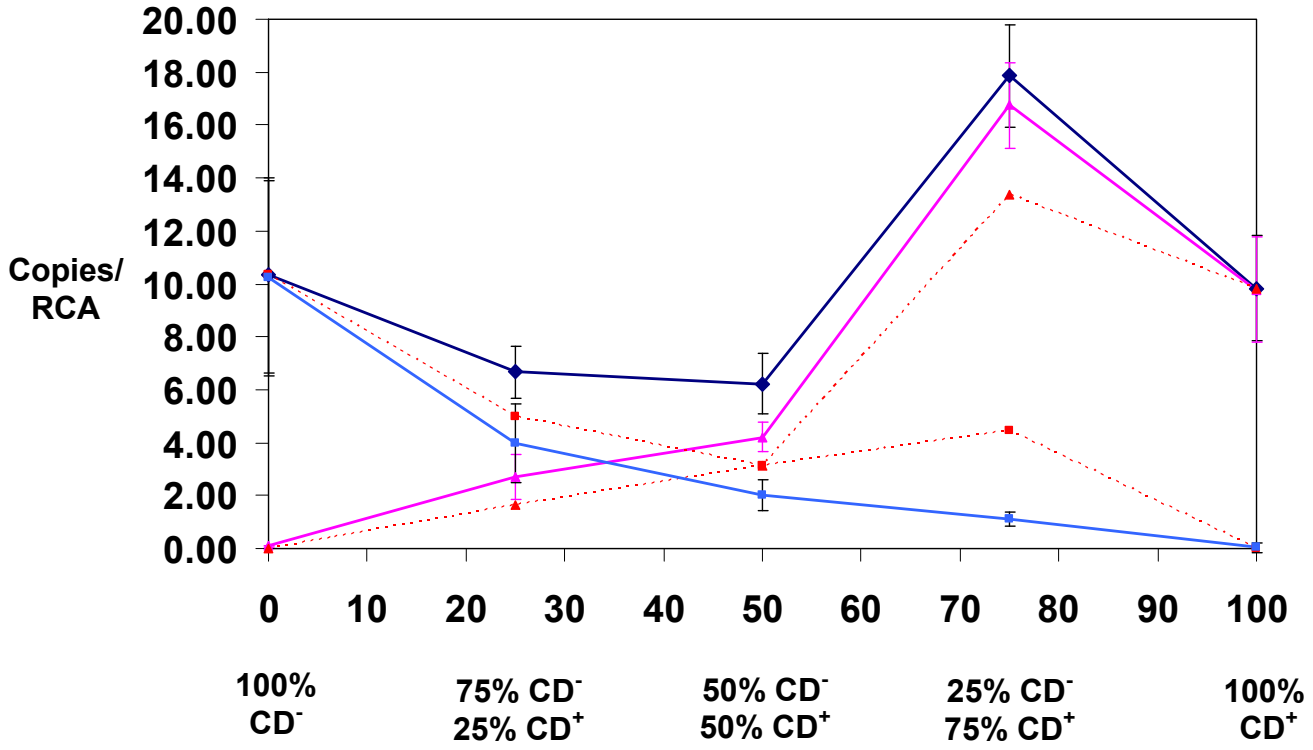
Species	Cultivar	Component	Plant age	Major components			Reference(s)
				Organic acids	Sugars	Amino acids	
M. sativa	Regen-SY	Root exudate	3 weeks	acetic acid, succinic acid, malic acid, citric acid			Tesfaye et al. 2001
M. sativa	GA-AT	Root exudate	3 weeks	malic acid citric acid, succinic acid			Tesfaye et al. 2001
M. sativa	Europe	Root exudate	5 weeks	citric acid, malic acid, malonic acid			Fougere et al (1991)
M. sativa	Europe	Root extract	5 weeks		sucrose, pinitol, glucose, fructose		Fougere et al (1991)
M. sativa	Moapa 69	Root exudate	8 days		fructose, sucrose, mannose, glucose		El-Hamalawi and Erwin (1986)
M. truncatula	Jemalong	Root nodule extract	12 weeks		sucrose, fructose		Lopez et al. 2008
M. truncatula	Jemalong A17	Root exudate	5 days			serine, alanine, proline, valine, aspartate	Phillips et al. (2004)
M. sativa	Moapa 69	Root exudate	8 days			phosphoserine, aspartate, citrulline, asparagine, alanine	El-Hamalawi and Erwin (1986)
M. sativa	Verko	Root exudate	3 weeks			lysine, glutamine, aspartate, histidine, arginine.	Miersch et al. (1992)

Supplemental Table B.5. Components of tomato root exudates as cited in the literature.

Cultivar	Component	Plant age	Major components			Reference(s)
			Organic acids	Sugars	Amino acids	
Karmello	root exudates	2 weeks	citric acid, succinic acid, glutamic acid, malic acid			Kamilova et al. (2006)
Karmello	root exudates	2 weeks	citric acid, malic acid, lactic acid, succinic acid, oxalic acid, pyruvic acid			Kravchenko et al. (2003)
Karmello	root exudates	2 weeks		glucose, xylose, fructose, maltose, sucrose		Lugtenberg et al. (1999)
Karmello	root exudates	2 weeks		xylose, maltose, glucose, fructose, sucrose		Kravchenko et al. (2003)
South Australia Early Dwarf	root exudates	2 weeks			glutamic acid, aspartic acid, serine, glutamine, asparagine, glycine, alanine	Rovira (1959)
Karmello	root exudates	7 days			glutamic acid, aspartic acid, leucine, isoleucine, lysine, serine/asparagine, glycine/glutamine, phenylalanine, arginine/threonine	Simons et al. (1997)

Supplemental Table B.6. Results of *N. haematococca* MPVI substrate utilization analysis using various organic acids, sugars, and amino acids.

Nutrient source	77-13-4	HT1	HT5	230-30-6	230-CL-3
Citric acid	sparse	sparse	sparse	sparse	sparse
Succinic acid	+	+	+	+	+
Malic acid	+	+	+	+	+
D-Glucuronic acid	sparse	sparse	sparse	sparse	sparse
α-D-Galacturonic acid	+	+	+	+	+
Xylose	compact	compact	compact	compact	compact
Pinitol	+	+	+	+	+
Phosphoserine	+	+	+	+	+
Citrulline	+	+	+	+	+
DL-Isoleucine	+	+	+	+	+
L-Isoleucine	+	+	+	+	+
DL-Lysine	+	+	+	+	+
L-Lysine	+	+	+	+	+
Allantoin	very sparse	very sparse	very sparse	very sparse	very sparse
Control M-100	+	+	+	+	+



Supplemental Figure B.1. The deWit Replacement Diagram of data for the repeat experiment with pea inoculated with Tr78.2:HT5. As shown in the original experiment with these two isolates, there is better pea rhizosphere colonization by the isolate containing a portion of the *PDA1* CD chromosome (Tr78.2; pink line) than by the isolate without that portion of the CD chromosome (HT5, blue line), as indicated by a higher-than-expected ratio of *PDA1* relative to *actin* gene. Some of the values obtained for RCA, however, are higher by as much as three-fold when compared to those obtained in the original experiment.

APPENDIX C: IDENTIFICATION OF HOMOSERINE UTILIZATION GENES IN *NECTRIA HAEMATOCOCCA* MATING POPULATION VI (MPVI) AND THEIR CONTRIBUTION TO RHIZOSPHERE COMPETENCY

Abstract

The fungus *Nectria haematococca* MPVI is a habitat-diverse fungus and habitat-determining genes in this fungus can be found on supernumerary chromosomes called "conditionally dispensable" (CD) chromosomes. Previous work has shown that the *N. haematococca PDA1* CD chromosome carries genes for the utilization of homoserine, an amino acid found in large quantities in pea root exudates. Furthermore, a *N. haematococca* MPVI isolate that has a portion of this CD chromosome containing the genes for homoserine utilization (HUT) is more competitive in the pea rhizosphere than an isolate without the CD chromosome. In the current study, a combination of fungal transformation, insertional mutagenesis, and bioinformatics analyses were used to locate and identify a cluster of five genes on the *PDA1* CD chromosome that were responsible for the HUT phenotype. One of the genes was only found in *N. haematococca* MPVI, another was a common fungal transcription factor and the other three had homologs involved in the synthesis of the amino acids methionine, threonine, and isoleucine, in which homoserine is an intermediate. Competition experiments between isolates with and without the *HUT* cluster demonstrated that the *HUT* cluster is responsible for the increased competitive ability of HUT^+ *Nectria haematococca* isolates in the rhizosphere of pea plants. To our knowledge, this is the first study to identify a rhizosphere competency trait in a fungus.

Introduction

Various experimental strategies have identified several bacterial traits responsible for rhizosphere-competence and colonization (Rosenberg et al. 1981, Boivin et al. 1991, Baldani et al. 1992, Goldmann et al. 1991, 1994, Martinez-Romero and Caballero-Mellado 1996, Phillips et al. 1996, Lugtenberg et al. 2001). For example, studies on the symbiotic N-fixing bacteria in legume roots have demonstrated that the genes for nodulating (NOD genes) specific hosts are found on the sym (symbiosis) plasmid of *Rhizobium leguminosarum* (Rosenberg et al. 1981, Martinez-Romero and Caballero-Mellado 1996) and these same plasmids can also carry genes for catabolizing unique compounds present in the root exudates of the host plants (Rosenberg et al. 1981, Boivin et al. 1991, Goldmann et al. 1994). The correlation between nodulation specificity and the ability to catabolize specific host root exudates was originally proposed over thirty years ago, when it was found that a pea-nodulating strain of the bacterium *R. leguminosarum* biovar *viciae* could catabolize homoserine (HS), a compound found in high concentrations in pea (*Pisum sativum* L.) root exudates (VanEgeraat 1975a and 1975c). The sym plasmid of *R. leguminosarum* biovar *viciae* was later shown to carry the gene for homoserine utilization as well as the NOD genes (Economou et al. 1988).

In Appendix A of this work it was shown that the fungus *Nectria haematococca* MPVI, a root rot pathogen of pea, can also utilize the amino acid homoserine as a sole carbon and nitrogen source (Rodriguez et al. 2008). It was also shown that the gene(s) for this trait reside on a conditionally dispensable (CD) chromosome. CD chromosomes are supernumerary chromosomes that are not needed for axenic growth but contain genes that

allow growth in different habitats (Covert 1998). I showed that the *PDA1* CD chromosome that contains the gene(s) conferring the HS-utilization phenotype also gave *N. haematococca* MPVI an increased competitive advantage in the pea rhizosphere (Rodriguez et al. 2008, Appendix A).

In the current study I use a combination of fungal transformation, insertional mutagenesis, and bioinformatics analyses to specifically locate and identify the genes on the *Nectria haematococca* MPVI *PDA1* CD chromosome that are responsible for the HS-utilization (HUT) phenotype. I also use real-time PCR to determine if the identified genes contribute to an increased competitive ability of *Nectria haematococca* in the rhizosphere of pea plants.

Materials and Methods

Fungal strains and media

N. haematococca MPVI isolates 77-13-4, Tr78.2, and HT-5 and *F. oxysporum* f. sp. *lycopersici* isolate NRRL 26383 were obtained from the culture collection of Dr. Hans VanEtten. Isolate 77-13-4 contains the *PDA1* CD chromosome, is pathogenic on pea, and is HUT^+. Tr78.2 contain a portion of the *PDA1* CD chromosome that has the HUT gene(s) but lacks the Pea Pathogenicity (*PEP*) gene cluster and is not pathogenic on pea. HT5 lacks the *PDA1* CD chromosome and therefore is not pathogenic on pea and is HUT^-. *Magnaporthe grisea* isolate 70-15 and *Neurospora crassa* isolate 74A were obtained from the culture collection of Dr. Marc Orbach. Stock cultures of *N. haematococca* MPVI were maintained as slant cultures on V-8 agar medium containing,

per liter, 200 ml V8® juice, 3.0 g $CaCO_3$, and 20 g agar (Stephens 1974). The stock culture of *N. crassa* was maintained on Vogel's agar medium (Davis and de Serres 1970), containing, per liter, 15 g sucrose and 20 ml Vogel's 50X Salts (containing, per liter 150 g Na_3 citrate•$5H_2O$, 250 g KH_2PO_4, 100 g NH_4NO_3, 10 g $MgSO_4$•$7H_2O$, 5 g $CaCl_2$·$2H_2O$, 5 mL trace elements [containing, per 100 ml, 5 g citric acid•H_2O, 5 g $ZnSO_4$•$7H_2O$, 1 g $Fe(NH_4)_2SO_4$•$6H_2O$, 250 mg $CuSO_4$•$5H_2O$, 50 mg $MnSO_4$•H_2O, 50 mg H_3BO_3, 50 mg Na_2MoO_4•$2H_2O$, and 1 ml chloroform], and 2.5 ml biotin solution (100 mg/L). The stock culture of *M. grisea* was maintained on oatmeal agar (Crawford et al. 1986) containing 30 g oatmeal per liter of water. Cultures were incubated in the dark at 24±1°C.

M-100 minimal medium (Stephens 1974), containing, per liter, 10 g glucose, 3 g KNO4, 20 g agar, and 62.5 ml M-100 salt solution (containing, per liter, 16 g KH_2PO_4, 4 g Na_2SO_4, 8 g KCl, 2 g $MgSO_4$•$7H_2O$, 1 g $CaCl_2$, and 8 ml trace elements) was amended with 65 mg hygromycin-B (HygB; Calbiochem, La Jolla, CA) and used to culture newly isolated transformants.

Modified M-100 minimal medium, containing, per liter, 20 g DL-homoserine (HS; MP Biomedicals LLC, Solon, OH) as the sole carbon and nitrogen source, 3 g Gelrite® Gellan Gum (Sigma-Aldrich Co., St. Louis, MO), and 62.5 ml M-100 Salt Solution, was used to determine if *N. haematococca* isolates could utilize HS as a sole carbon and nitrogen source and for growth measurement of transformants on solid medium. The same medium, without Gelrite® Gellan Gum, was used for growth measurement of transformants in liquid medium.

Spore production and collection

N. haematococca MPVI spores were produced in Petri dishes containing solidified V-8 agar medium (Stephens 1974). CD^{+} [1] and CD^{-} isolates used in this study had been previously cultured and examined to ensure that they had similar growth rates and production of micro- and macroconidia. Cultures were incubated at 24±1°C under lighted conditions to encourage production of macroconidia. Generally, cultures grown under these conditions gave similar percentages (~80-100%) of macroconidia (data not shown). After 1-2 wks of growth, the spores were harvested, rinsed with sterile water, suspended in water, and counted using a hemacytometer. For the rhizosphere competence assays the number of viable spores in the inocula was confirmed by dilution plating the spore suspensions on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) and counting fungal colonies. For inoculation of rhizospheres, macroconidia were counted as five cells since the vast majority of macroconidia produced by the *N. haematococca* isolates used had five nuclei, whereas microconidia were counted as single cells. For the purpose of spore count confirmation via culturing on PDA, both micro- and macroconidia were counted as single spores.

Preparation of cosmids

N. haematococca MPVI isolate 77-13-4 has been sequenced and candidate genes on the *PDA1*-CD chromosome for HS utilization were identified (Rodriguez-Carres 2006). High-molecular-weight genomic DNA from *N. haematococca* MPVI isolate 77-13-7 was previously used to construct an ordered genomic library (Rodriguez-Carres

[1] CD^{+} = containing the CD chromosome; CD^{-} = not containing the CD chromosome

2006) in the cosmid vector pMOcosX (Orbach 1994). Ends of the cosmids in the ordered genomic library for the CD chromosome were previously sequenced (Rodriguez-Carres 2006), thereby allowing for the identification of the cosmids (2B07, 2F03, and 5B04) with open reading frames (ORFs) suspected to be involved in HS utilization. Cosmid DNA was prepared using a standard alkali lysis protocol and purified by phenol/chloroform extraction (Sambrook et al. 1989).

Transformation of *Nectria haematococca* and *Fusarium oxysporum* f. sp. *lycopersici*

Transformation of *N. haematococca* and *F. oxysporum* f. sp. *lycopersici* was accomplished using a procedure modified from Sweigard et al. (1992). The CD^- *N. haematococca* MPVI isolate HT5 and the *F. oxysporum* f. sp. *lycopersici* isolate NRRL 26383, which are both negative for HS utilization, were streaked onto V8 juice agar (Stephens 1974) in Petri plates and the plates incubated at 25°C for 7 days under light for spore production. Mycelia for protoplast formation were produced by harvesting spores from single V8 agar plates and adding them to 100 ml of Difco™ potato dextrose liquid medium (Becton, Dickinson, and Co., Sparks, MD) followed by incubation on a rotary shaker (200 rpm) at room temperature overnight. The resulting mycelia were harvested by filtration, washed with distilled water, weighed, and 1 g of each mycelium was resuspended in 15 ml of 0.7 M NaCl. Kitalase (Wako Pure Chemical Industries Ltd., Richmond, VA) and Driselase (InterSpex Products, Inc., San Matea, CA) or Lytizyme (Karlan Research Products Corp., Cottonwood, AZ) were then added at a rate of 6 mg (for Kitalase) and 5 mg (for Driselase and Lytizyme) per ml of suspension. The enzyme/mycelium mixtures were gently mixed at room temperature. After 2-2.5 hr,

protoplasts were harvested by filtering through sterile Miracloth (pore size: 22-25 µm; Calbiochem, San Diego, CA). The protoplast suspensions were centrifuged in a swinging bucket rotor (2000 rpm) for 10 min. and the pellets were resuspended in 10 ml of 0.7 M NaCl. This step was repeated and the protoplasts were resuspended in 10ml of SuTC (20% sucrose, 50 mM TRIS-HC1 pH 8.0, 50 mM $CaC1_2$).

The protoplasts were counted using a hemacytometer, then centrifuged as before and resuspended in SuTC to obtain a concentration of 1 x 10^7 spores/ml. Protoplasts (0.1 ml) were mixed with 15 µg DNA (suspended in SuTC containing 20% sucrose, 50 mM TRIS-HC1, pH 8.0, and 50 mM $CaC1_2$), 0.5 µl 20mM aurintricarboxylic acid (ATA; Sigma Corp., St. Louis, MO) and 0.5 µl 50mM spermadine (Sigma Corp., St. Louis, MO). After 20 min incubation on ice, 1 ml PEG solution (60% polyethylene glycol 3500, 20% sucrose, 50mM Tris pH 8.0, 50mM $CaCl_2$) was added. After an additional incubation for 25 min., 3 ml of TB3 medium (20% sucrose, 1% glucose, 0.3% yeast extract, and 0.3% casamino acids) was added, and the mixtures were gently mixed 20-22 h at room temperature. Following incubation, the mixtures were centrifuged as before and the supernatants were discarded. Eight ml of molten regeneration medium (20% sucrose, 1% glucose, 0.3% yeast extract, 0.3% casamino acids, 1.5% agarose) at 50°C were added, and the protoplasts were poured onto Difco™ potato dextrose agar (Becton, Dickinson, and Co., Sparks, MD) plates containing sufficient hygromycin B (HygB) for a final concentration of 65 µg/ml. Plates were incubated at room temperature, with transformants usually appearing in 3-7 days. Transformants were picked to M-100 agar plates containing 65 µg/ml of HygB. Transformants that grew on M-100 agar with HygB

were tested for their ability to use HS as a sole carbon and nitrogen source. All transformants were stored as slant cultures on V8 medium and as glycerol stock cultures at -80^{o}C.

HS growth assay

Radial growth of putative transformants was measured by inoculating modified M-100 medium containing HS with either mycelium or spores. After 3 days the medium was examined for hyphal growth.

Growth measurements of representative transformants, with either cosmid 2B07 or cosmid 2F03, were performed on solid and in liquid modified M-100 minimal medium with HS as a sole carbon and nitrogen source. For solid medium plates, 7.5 x 10^4 spores in a total volume of 10 µl were added to the center of the solid medium. Three plates were inoculated for each isolate and then incubated at room temperature. After 7 days the colony diameters were measured. For liquid media, 7.5 x 10^4 spores were inoculated into 15 ml of medium in 50 ml flasks (for a final concentration of 5000 spores/ml). Three flasks were inoculated for each isolate and then incubated with shaking at room temperature. After 5 days the mycelia were harvested by filtration, washed with distilled water, and lyophilized overnight. Following lyophilization, the dry weights of the mycelia were determined. All cultures were set up in triplicate and the experiment was repeated once.

Cosmid mutagenesis

Mutagenesis of genes on cosmid 2B07 via transposon insertions was accomplished using the GPS®-1 Genome Priming System (New England Biolabs Inc., Ipswich, MA), which disrupts genes by placing a transposon into target DNA at random locations. 0.03 µg of cosmid 2B07 DNA was mixed with 0.06 µg pGPS1.1 donor DNA, 2 µl 10X GPS buffer, and 1 µl TnsABC transposase in a total volume of 19 µl and incubated at 37°C for 10 min. After addition of 1 µl of Start Solution, the reaction mixture was incubated at 37°C for 1 hr and then heat-inactivated at 75°C for 10 min.

Transformation of *E. coli* cells with mutagenized cosmid 2B07 was performed according to standard protocol. In brief, 1 µl of the mutagenesis reaction mixture was added to 50 µl of *E. coli* DH5α cells (10^7 cells/ml concentration). The transformation mixture was incubated on ice for 30 min. and then heat-shocked at 42°C for 45 sec. After addition of 750 µl LB broth, the mixture was incubated at 37°C for 30 min. Cells were concentrated by centrifugation for 2 min. and 10 µl of cells were plated onto LB agar containing kanamycin (20 µg/ml) for selection of transformants.

Cosmid DNA was extracted from kanamycin-resistant transformants using a standard mini-prep protocol (Sambrook et al. 1989). Transposon insertions were located by DNA sequencing using the following primers from the GPS®-1 Genome Priming System: Primer S, 5'-ATAATCCTTAAAAACTCCATTTCCACCCCT-3' and Primer N, 5'-ACTTTATTGTCATAGTTTAGATCTATTTTG-3'. Cosmid DNA from cosmids with transposons inserted in the putative HS utilization genes was prepared as described above and used for transformation of *N. haematococca* isolate HT5.

Bioinformatic Analyses

Putative proteins encoded by genes on the overlapping region of cosmids 2B07 and 2F03 were identified using InterProScan (European Bioinformatics Institute; http://www.ebi.ac.uk/Tools/InterProScan/). Using the NCBI BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) putative protein identifications were verified based on homology with proteins (or putative proteins) of other organisms. The *N. haematococca* MPVI v2.0 genome sequence (http://genome.jgi-psf.org/Necha2/Necha2.home.html) was used to determine the presence of *PDA1* CD chromosome genes on other chromosomes in the *N. haematococca* MPVI genome. The European Bioinformatics Institute ClustalW2 sequence alignment program (http://www.ebi.ac.uk/Tools/clustalw2/) was used for sequence alignment of putative proteins encoded by CD chromosome genes with hypothetical and known proteins identified by the NCBI BLAST search.

Rhizosphere Competition Assays

The replacement series technique (deWit 1960), as described in Appendices A and B, was used to measure rhizosphere competition as a function of the relative abundance of two competing *N. haematococca* MPVI isolates in the rhizosphere of pea plants. In order to evaluate the competitive advantage conferred on *N. haematococca* MPVI by HUT, competition experiments were done between (1) HT5 and transformant HT5-2B07, (2) Tr78.2 and transformant HT5-2B07, and (3) HT5 and transformant HT5-2B07Δ3.

Seeds of pea (cv. Little Marvel; Royal Seeds, Kansas City, MO) were surface-sterilized in 70% ethanol for 5 min., in a 2.5% sodium hypochlorite solution for 10 min., and then rinsed thoroughly with sterile distilled water. Surface-sterilized pea seeds were planted, one seed per jar, in 1-quart Mason jars containing 300 g of sterile potting mixture (four parts vermiculite to one part quartz sand wetted with 1 L of sterile distilled water per 10 L of mixture).

Germination of seeds, as indicated by the appearance of the radical, generally took place within 1-5 days. Following germination, plants were grown under a 12-hour light, 12-hour dark regime at 24±1^{o}C temperature. After a two-week growth period, the potting mixture was inoculated with spores of two *N. haematococca* MPVI isolates as described above. The final spore concentration was prepared to give a constant density of 10^5 *N. haematococca* MPVI spores per gram of potting mixture but at varying ratios (0:100, 25:75, 50:50, 75:25, and 100:0) of the two isolates. The plants were grown for another three weeks under the same conditions. During the growth period, the plants were watered with Hoagland's solution every 4-6 days. At the end of the 2-4 wk period, the rhizospheres and roots were harvested by cutting the stem immediately above the uppermost roots and gently shaking off the loose potting mixture. The harvested roots and adhering potting mixture were lyophilized and ground to a fine powder in liquid nitrogen using a mortar and pestle. DNA for real-time PCR was extracted from one gram of ground rhizosphere material using the Ultraclean™ Soil DNA Kit (Mo Bio Laboratories, Inc., Solana Beach CA). Three replicates were performed for each ratio of *N. haematococca* MPVI isolates and all experiments were repeated.

Real-time quantitative RT-PCR

The number of cells in each sample was determined from the target gene copy number, which was quantified by comparing the Ct value of the samples to the Ct value of the respective standard curve. Standard curves for the fungal marker genes were constructed with serial dilutions of the PCR products obtained from the genomic DNA of *N. haematococca* MPVI isolate 77-13-4, which has the *PDA1* CD chromosome or *N. haematococca* MPVI transformant Tr78.2, which contains the hygromycin resistance gene (Wasmann and VanEtten 1996). The standard curve for the plant gene rubisco activase (*RCA*) was constructed with serial dilutions of the PCR products obtained from the genomic DNA of pea (cv. Little Marvel). PCR products for standard curves were produced by using the same primer pairs as those used for the real-time quantitative PCR of rhizosphere samples. The *N. haematococca* MPVI actin gene (Liu et al. 2003) was used for real-time PCR to determine the total number of *N. haematococca* MPVI cells in the rhizosphere samples. The hygromycin resistance gene (*hph*), which is on the cosmid vector pMOcosX (Orbach 1994) was used for real-time PCR to determine the total number of transformant HT5-2B07 and transformant HT5-2B07Δ3 cells in the rhizosphere samples of experiment 1 (HT5 vs. HT5-2B07) and experiment 3 (HT5 vs. HT5-2B07Δ3), respectively. The *N. haematococca* MPVI *PDA1* gene (Maloney and VanEtten 1994), which is on the *PDA1* CD chromosome, was used for real-time PCR to determine the number of transformant Tr78.2 cells in the rhizosphere samples of experiment 2 (Tr78.2 vs. HT5-2B07). Tr78.2 lacks a wild-type copy of *PDA1* but contains the hygromycin resistance gene (*hph*) flanked by 692bp 5' and 888bp 3' of the

PDA1 gene (Wasmann and VanEtten 1996). The portion of *PDA1* from the transformation knockout vector was used in this study as a marker for Tr78.2. Transformant Tr78.2 also carries a portion of the *PDA1* CD chromosome that contains the *HUT* gene(s) (Rodriguez et al. 2008, Appendix A). Real-time quantitative PCR using TaqMan® technology was performed on the Abbott Prism® System (Abbott Park, Illinois) according to the manufacturer's protocol. Sequences of the primers (Invitrogen Corporation, Carlsbad, California) and TaqMan® fluorescent probes (Applied Biosystems, Foster City, CA) used in the quantitative real-time PCR study are listed in Table C.1.

Each TaqMan® probe was designed to anneal to a specific sequence between the forward and reverse primers of its target gene and to have at least a 5^{o}C higher T_m than the PCR primers. Individual probes contain a reporter fluorochrome (6-carboxyfluorescein [6-FAM] for *PDA*-1 and actin, tetrachloro-6-carboxyfluorescein [TET] for *RCA*) at the 5' end and a quencher fluorochrome (6-carboxy-tetramethyl-rhodamine [TAMRA]) at the 3' end. PCR analyses were performed in duplicate, with duplicate reactions on each run. Each reaction had a total volume of 25 µl containing 12.5 µl of either qPCR® Mastermix Plus (for *PDA1*, actin, and *RCA*; Invitrogen Corp., Carlsbad, CA; contains reaction buffer dNTPs (including dUTP), Hot Goldstar® DNA polymerase, $MgCl_2$, and uracil-N-glycosylase), or Platinum® SYBR® Green qPCR SuperMix-UDG (for *hph*; Invitrogen Corp., Carlsbad, CA; contains reaction buffer,

Table C.1. Primers and probes used in this study.

***Nectria haematococca* primers and probes used for rhizosphere experiments**	
Actin forward primer	5'-ATCCACG- TCACCACCTTCAA-3'
Actin reverse primer	5'-GTGCCAGAGTTAGAAATGATC-3'
Actin probe	FAM-ACATCGACATCACACTTCATGATGGAG-TAMRA
PDA1 forward primer	5'-GATGAGCAGACTGAGGTTGGT-3'
PDA1 reverse primer	5'-CTGTGATGCCAAGGTCACTTA-3'
PDA1 probe	FAM-TGCCTGCAGTGCCCTGGACG-TAMRA
Hyg^R (*hph*) forward primer	5′-CTGTCGAGAAGTTTCTGATCG-3'
Hyg^R (*hph*) reverse primer	5′-GGAAGTGCTTGACATTGGG-3'
Primers and probes used for Pea	
RCA forward primer	5'- CTTGTTCATCAACGATCTTGAT-3'
RCA reverse primer	5'-GGTTGTCAGCAATGTTCATGAG-3'
RCA probe	TET-CACCGTCAACAACCAGATGGTGAATG-TAMRA

dNTPs, Platinum® *Taq* DNA Polymerase, $MgCl_2$, uracil-N-glycosylase, and SYBR® Green I), 300 nM forward primer, 300 nM reverse primer, 200 nM Taqman® probe (for *PDA1*, actin, and *RCA*; SYBR Green was used for real-time PCR amplification of *hph* and it is included in the Platinum® SYBR® Green qPCR SuperMix-UDG), 2 µl rhizosphere DNA template, and 9 µl of sterile distilled water. The PCR parameters were as follows: an initial uracil-N-glycosylase step at 50°C for 2 min, followed by an initial denaturation and Hot Goldstar® DNA polymerase activation step at 95°C for 10 min., and 40 cycles at 95°C for 15 sec. and 60°C for 30 sec. Real-time PCR for each gene was performed at least three times.

Non-inoculated plants and no-plant inoculated soils harvested at the end of the experiment served as controls for these experiments.

deWit Replacement Curves

As described in Appendix A of this study, the *RCA* gene from pea was used to measure the number of corresponding plant cells in samples and to normalize the amount and quality of the genomic DNA.

Competition between different isolates was shown diagrammatically by plotting the results on a deWit replacement curve, on which dashed lines represent the growth of the isolates without competition and solid lines represent the actual growth under competition. If there is no competition between the two isolates, i.e., each can colonize the rhizosphere equally, then the ratios of the two isolates recovered from the rhizosphere of plants inoculated with mixtures of two different isolates should be the same as the inoculation ratios.

Transformation of *Magnaporthe grisea*

Transformation of *Magnaporthe grisea* was performed using a procedure modified from Sweigard et al. (1992). *M. grisea* isolate 70-15 protoplasts (a gift from Dr. M.J. Orbach) were prepared as previously described (Sweigard et al. (1992). For transformation, 200 µl of protoplasts (~10^7) were mixed with 1-5 µg of DNA suspended in 15 µl of SUTC and incubated at RT for 15 min. One ml of PEG solution (containing 40% 8000 MW polyethylene glycol, 20% sucrose, 50mM Tris, and 50mM $CaCl_2$) was added. The suspension was incubated for 20 min. at RT and 3 ml of TB3 (20% sucrose, 1% glucose, 0.3% yeast extract, and 0.3% casamino acids) were added. The suspension was mixed by inversion, incubated overnight with gentle rocking on a rocking platform, and centrifuged at 3000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in 100 µl of SUTC. Eight ml of molten regeneration medium (containing TB3 with 1.5% agarose) at 50°C were added to the transformed cells. The suspension was mixed by gentle inversion and poured onto complete regeneration medium (containing 20% sucrose, 1% glucose, 0.3% yeast extract, 0.3% casamino acids, 1.7% agar, and 300 µg/ml of hygromycin). Plates were incubated at 28°C for 4-5 days before picking hygromycin-resistant transformants.

Transformation of *Neurospora crassa*

Transformation was accomplished using a procedure modified from Orbach et al. (1986). *N. crassa* isolate 74A protoplasts (a gift from Dr. J. Coleman) were prepared as previously described (Orbach et al 1986). For transformation, 5 µg cosmid DNA in a volume of 10 µl was mixed with 15 µl of heparin solution (5 mg heparin per ml of STC)

and 100 µl (~10^7) of spheroplasts were added. Followed by incubation on ice for 30 min., 1 ml of 60% PEG solution was added, and the suspension was mixed by inversion. After incubating for 20 min. at room temperature, 8 ml of molten regeneration top agar (containing, per liter, 20 ml 50X Vogel's salts solution, 182 g sorbitol, 1 ml biotin solution, 1 ml trace elements solution, and 15 g agar) at 50°C were added to the transformed cells. The suspension was mixed by gentle inversion and poured onto regeneration medium (containing, per liter, 20 ml 50X Vogel's salts solution, 1 ml biotin solution, 1 ml trace elements solution, 15 g agar, 100 ml 10X sugars solution [containing 20% sorbose, 0.5% fructose, 0.5% glucose, and 0.2% myo-inositol] and 200 µg/ml of hygromycin). Plates were incubated at 30°C for 2-7 days before picking hygromycin-resistant transformants.

Results

Transformation of *Nectria haematococca*

Cosmids 2B07 and 2F03 from a *PDA1*-CD chromosome specific library (Rodriguez-Carres 2006) were chosen for transformation of *N. haematococca* because they had predicted ORFs that might be expected to be involved with HS utilization (e.g. HS dehydrogenase). Transformation of *N. haematococca* isolate HT-5, which lacks the *PDA1*-CD chromosome (chromosome 14) and is negative for HS utilization, with cosmids 2B07 and 2F03 yielded nine and fifteen hygromycin-resistant transformants respectively. All of these transformants could use HS as a sole C and N source. No transformants were obtained with cosmid 5B04.

Growth measurements of two representative transformants, HT5-2B07-1 and HT5-2F03-1, were performed on solid and in liquid modified M-100 minimal medium with HS as a sole carbon and nitrogen source. The mean colony diameters for *N. haematococca* isolate 77-13-4 (CD^+), and transformants HT5-2B07-1 and HT5-2F03-1 were 56.7, 56.0, and 56.7 mm after growth on solid HS medium for 7 days. Dry weights of mycelia of *N. haematococca* isolate 77-13-4 (CD^+), and transformants HT5-2B07-1 and HT5-2F03-1 were 46.8, 46.7, and 43.5 mg after growth in liquid HS medium for 5 days.

Bioinformatic analyses

The flanking ends of the inserted genomic DNA of cosmids 2B07 and 2F03 were previously sequenced (M. Rodriguez, unpublished). These end sequences flanking the insertion sites of cosmid vector pMOcosX were used to identify the sequence of the inserted genomic DNA. Bioinformatic analyses using the BLAST program showed that cosmids 2B07 and 2F03 have an over-lapping region of 13.3 Kb (Figure C.1). Within this 13.3 Kb region the following five putative proteins were identified using InterProScan (European Bioinformatics Institute; http://www.ebi.ac.uk/Tools/InterProScan/): 1 = unknown protein, 2 = fungal transcriptional regulatory protein, 3 = β-aspartate-semialdehyde dehydrogenase (succinate-semialdehyde dehydrogenase), 4 = HS dehydrogenase, 5 = cystathionine-β-lyase/cystathionine-γ-synthase. Using the NCBI BLAST search these putative proteins were verified based on homology with proteins in other organisms.

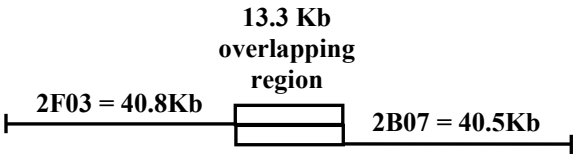


Figure C.1. 13.3 Kb overlapping region of cosmids 2B07 and 2F03.

Transformation with mutagenized cosmids

Disruption of genes 1-5 on cosmid 2B07 via pGPS1.1 transposon mutagenesis yielded at least two disruptions for each of the five genes (data not shown). Subsequent transformation of HT5 with any of the mutated cosmids did not restore the HS utilization phenotype (Figs. C.2, C.3, and C.4).

Using the *N. haematococca* v2.0 genome sequence it was found that the only copy of gene 1 is on chromosome 14 (i.e., the *PDA1* CD chromosome), whereas gene 2 is located on chromosomes 14 and 10, and genes 3, 4, and 5 are located on chromosomes 14 and 4. Genes 3 and 5 are located on one strand and gene 4 is located on the complementary strand (i.e. coding in the opposite direction) on chromosomes 4 and 14 (Fig C.5). Additional copies of genes 3 and 4 are located on chromosome 14 (also on scaffold 24 but ~95 Kb away) and chromosome 2 (scaffold 18), respectively.

Gene 1 was ~2000 bp and had five putative exons encoding ~672 amino acids (data not shown). Using the NCBI BLAST search the putative protein encoded by gene 1 was found to show highest identity with hypothetical proteins of *Magnaporthe grisea* (30% identity) and *Aspergillus fumigatus* (22% identity), and a putative Hsp70 family chaperone of *Aspergillus fumigatus* (22% identity).

Gene 2 on the CD chromosome had two putative introns. Clustal alignment of ~2500 bp region encompassing gene 2 on the CD chromosome (Chromosome 14, scaffold 24) and a normal chromosome (Chromosome 10, scaffold 82), showed 90% identity at the nucleic acid level (Fig. C.6); the open reading frames of gene 2 on the CD chromosome and normal chromosome showed 88% identity at the nucleic acid level (data

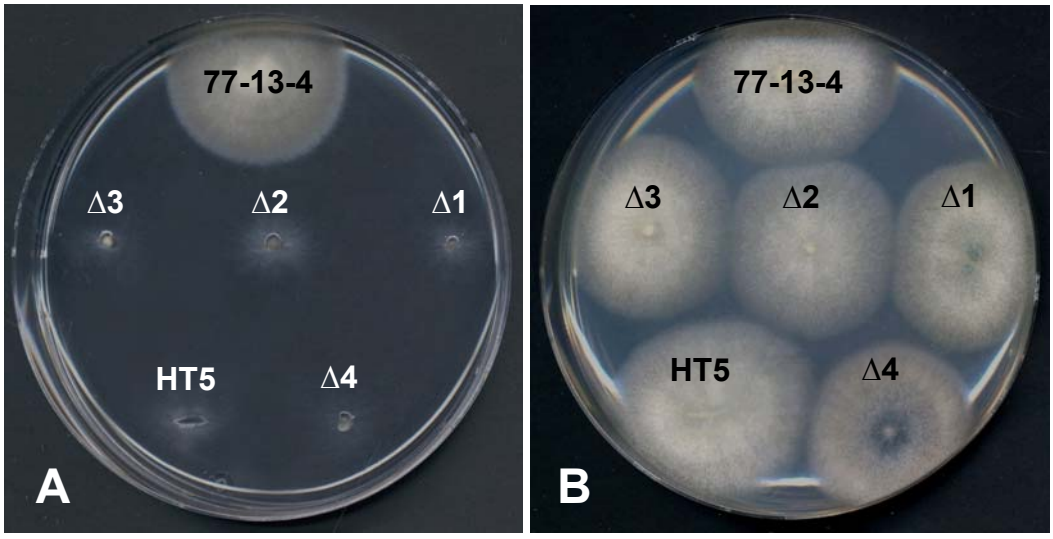


Figure C.2. *Nectria haematococca* 4-days post-inoculation on HS medium (A) and on M-100 medium (B). Isolate HT5 is CD^{-} and isolate 77-13-4 has the *PDA1* CD chromosome. HT5 transformed with cosmid 2B07 containing disrupted gene 3 (Δ3), disrupted gene 2 (Δ2), disrupted gene 1 (Δ1), and disrupted gene 4 (Δ4).

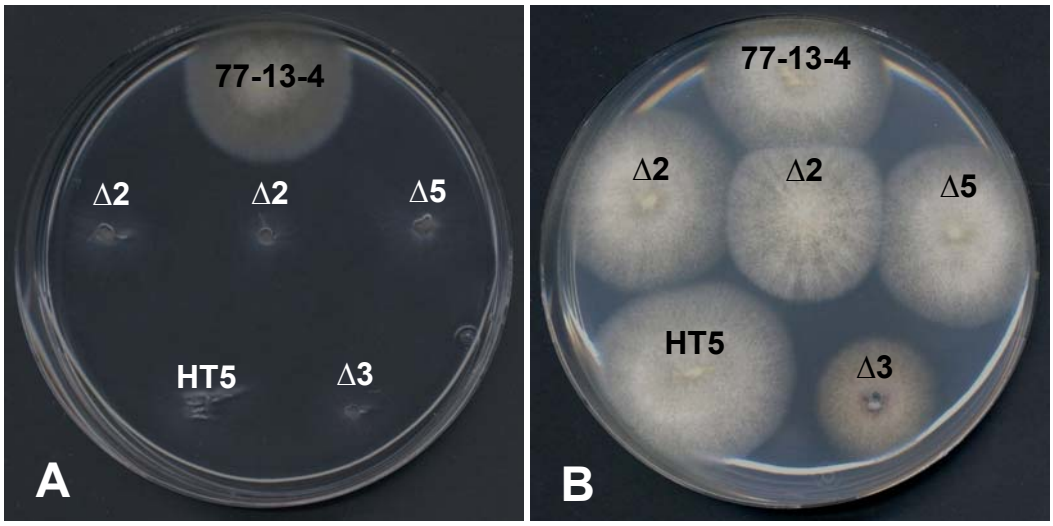


Figure C.3. *Nectria haematococca* 4-days post-inoculation on HS medium (A) and on M-100 medium (B). Isolate HT5 is CD^- and isolate 77-13-4 has the *PDA1* CD chromosome. HT5 transformed with cosmid 2B07 containing disrupted gene 2 (Δ2), disrupted gene 5 (Δ5), and disrupted gene 3 (Δ3).

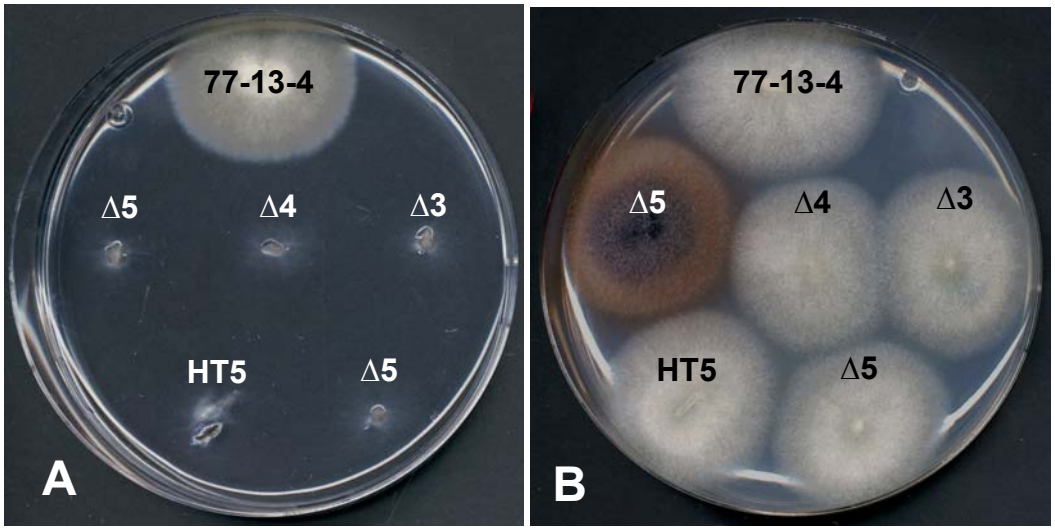


Figure C.4. *Nectria haematococca* 4-days post-inoculation on HS medium (A) and on M-100 medium (B). Isolate HT5 is CD^- and isolate 77-13-4 has the *PDA1* CD chromosome. HT5 transformed with cosmid 2B07 containing disrupted gene 5 (Δ5), disrupted gene 4 (Δ4), and disrupted gene 3 (Δ3).

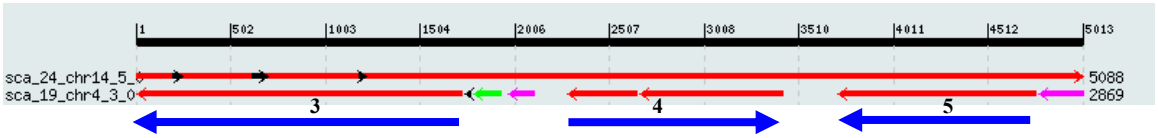


Figure C.5. Blast results of region containing genes 3, 4, and 5, showing that these genes are clustered on both chromosomes 14 and 4. Genes 3 and 5 are located on one strand and gene 4 located on the complementary strand of chromosome 4 and 14. Blue arrows show the orientation of these genes. Red indicates regions of greatest identity (alignment score >200), followed by pink (alignment score 80-200), and then green (alignment score 50-80). Note that the region containing the promoters for genes 3 and 4 is different on chromosomes 4 and 14 (as indicated by the green and pink arrows), as is the region containing the promoter for gene 5 (as indicated by the pink arrow).

```
gene2CD         ATTGGAGCCGATCCATCTTACTTTAGTCATCCTCCACAACTCGGTCTTCCGCTCGTCCTT 60
gene2endo       ------------------------------------------------------------

gene2CD         GCCGAGTACGGCTTAGAACCTCATCACGTCTTCCAACTTGATCCATCATCTCAGCTCTCG 120
gene2endo       ----------------ATCCTCATCACGTCTTCCAACTTGATTCATCATCTCAGGTCTCG 44
                               * ********************** ********** *****

gene2CD         GATCCTTCTGGCCATATCCGATCAATCAACCCTTATTGTGATCCATCACTGTTGTTATAC 180
gene2endo       GATCCTCCTGGCAATATCCGATCAATTAACCCTTATTGTGATCCATCACTGTTATAATAT 104
                ****** ***** ************* ************************ * ***

gene2CD         CCATGTCTGAAACACCAGAGCCTGAGGGCCAGCGACTTCGGCGTTCTATCGGAGTCTCAA 240
gene2endo       CCATGTCTGAAACACCAGAGCCTGAGGGCCAGCGACTTCGGCGCTCTTTCGGAGCCTCAA 164
                ****************************************** *** ****** *****

gene2CD          GGGCAACGCCGAAGAACGCACGCATTGCGTATGTCTACGACGAGTTGCCGGTTTCCAATG 300
gene2endo        GGGCAACGCCGAAGAACGCACGCATTGCGTATGTCCACGACGAGGTACCGGTTTCCAATG 224
                 ********************************** ******** * *************

gene2CD          AAATGGATGTTTACTAACTTTTTTAGTTGCCGAAAATGTCACGCTCGCAAAGTGAAATGC 360
gene2endo        AAATGGATCTCTACTAACTCTTTCAGTTGCCGAAAATGTCACGCCCGCAAAGTGAAATGC 284
                 ******** * ******** *** ******************** ***************

gene2CD          TCAGGCGGCACCCCCTGTGCCAACTGCCTTCAGGCCAATATCGGCGCTGAGTGCTCCTAT 420
gene2endo        TCTGGCGGTACTCCCTGTGCCAACTGCCTACAGGCCAATATTGGCGCTGAGTGTTCCTAT 344
                 ** ***** ** ***************** *********** *********** ******

gene2CD          CCAAGACGCATCAGGCGAGTGAAGGTCGATCAGAGGTATGGAAGCAAGGTAATAGTCCTT 480
gene2endo        CCAAGGCGAATCAGACGAGTGAAGGTCGATCAGAGGTATGAAAGCAAGTCATCAGTCCTT 404
                 ***** ** ***** ************************ *******  *  *******

gene2CD          TGGGATAACTACTAATCATGGGCTAGTTATATCGATGAACTGCTAGAAGAGATTCATACT 540
gene2endo        TAGGATAACTACTAATCATGGCCTAGTTATATCGATGAACTACTAGACGAGATTCATAGC 464
                 * ****************** ****************** ***** **********

gene2CD          TTTAGAAAAGCGGTTCATACCCCCCGAAGTTGCCAACTCAACATCGTCTCGGCGTCACGA 600
gene2endo        CTTAGAAAGGCTGTTCACACCCCC-GAAGCTGCCAACTCAACACCGTCTCGGCGTCACGA 523
                  ******* ** ***** ****** **** ************* ****************

gene2CD          GACCAGAGCTCTGTCTTTCGTCTCAGATGGCGATGCCGTCAACCAGGAATCGCCTGCCGC 660
gene2endo        GACAAGAGCCCAGTCTTTCGTCTCAGAAGGCGATGCCGTCAACCAGGAATCTCCTGCCGC 583
                 *** ***** * *************** ********************** ********

gene2CD          CTCTGTCATAAATGCTGACACGGGCGCATTGCAAGCCAATCCTGAAGCGACTGTCGTTGT 720
gene2endo        ATCTGTCATAAACGCCGATACGGGCATATTACAAGCCAATCCTGAAGCGACCGTCGTTGT 643
                  *********** ** ** ******  *** ******************** ********

gene2CD          TGATCAGACGAGACCTGCCCCAGAGACCGACGACTCTATCCGGAACCCGATCCTCGAGGA 780
gene2endo        TGACCAGACGAGACCTGCCCCAGAGACCGACGACTCTATCCGGAACCCGATTCTTGAGGA 703
                 *** ********************************************** ** *****

gene2CD          CCGTCCTTGGTTCTTCTCGCTGACCCCCGAGATGCCTATGCTTATAGAAGAAGCCGCAGA 840
gene2endo        CCGTCCTTGGTTCTTCTCGTTGACCCCCGAAATGCCTATGCTTATCGAGGAAGCCGCAGA 763
                 ******************* ********** ************** ** ***********

gene2CD          TGCACCATTCGCCACTAGATTCCGTCAAGAATTGTCTGGAAAATCACAACGACATATTCC 900
gene2endo        TGCACCTTTTGCTACCAGATTCCGTCAAGAACTCTCTGGGAAATCGCAACGACATATTCC 823
                 ****** ** ** ** *************** * ***** ***** **************

gene2CD          ACGCACCGAGAATGTGACCGATGAAGCATTAACATCGTCATGGAACACCCCTTGTCCCTG 960
gene2endo        ACGCACCGAAAACGTGACTGATGAAGCATTGACATCGTCATGGAACACACCTTGCCCATG 883
                 ********* ** ***** *********** ***************** ***** ** **

gene2CD          GCCGCCTGCATCAAGAGCCCGCTTCTTGCTCAAGGTGGCACTGAACACAGTATGCAAGCG 1020
gene2endo        GCCACCTGCATCAAGAGCCCGCTTCTTGCTAAAAGTGGCACTGAATACAGTATGCAAGCG 943
                 *** ************************** ** *********** **************
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```
gene2CD         CTACTATCTCGTTCGAAGGAGCGCTACACAGCGTCTACTGGAGCAAGCCATTCACAACCC 1080
gene2endo       CTATTATCTCGTTCGAAGGAGCGCTACGCAACGTTTACTGGAGCAAGTTATTCATAACCC 1003
                *** ********************** ** *** ************  ***** *****

gene2CD         CGACATGTGTGATCTTGTATCTGAGTACAAGCTCTTTGTACTGTTTGCATTGGGCGAGGT 1140
gene2endo       TGACATGTGTGATCTCGTATCTGAGTGCAAGCTCTTTGTCCTGTTTGCATTGGGCGAGGT 1063
                 ************** ********** ************ ********************

gene2CD         CTACTCGACGAGGACATCGCCTACCAAGGACAAGAATCAACTTCCTGGTATTTCATATTA 1200
gene2endo       TTACTCGACGAGGACATCGCCTACCAAGGACAAGAATCAACTTCCTGGTATCGCGTATTA 1123
                 ************************************************  * *****

gene2CD         TGTACGCGCCAGTCGGTTCCTTCGGGTCCTCACTGAACAACCTCGCATTGACTGCGTTGA 1260
gene2endo       TATACGCGCAAGCCGGTTCCTCCGGGTTCTCACTGAACAACCTCGGATCGACTGCGTTGA 1183
                * ******* ** ******** ***** ***************** ** ************

gene2CD         GGTTATCTTGATGCTGGTAAGGCTTTTGCTCTTGTCAGGGTTGCAATGC[TGATGCTAGAC] 1320
gene2endo       GGTTATCTTGATGCTGGTAAGACTTTTGGTCTTGTCAGT-TTGCAATGC[TAATACTCGAA] 1242
                ********************** ****** *********  ********** ** ** **

gene2CD         [AGTCTTTATACTCCCTGGCT]ATGAACCGACGGTACAACGCATACTGCATGATTGGATCTG 1380
gene2endo       [AGTCTTTATACTCTCTGGCT]ATGAACCGACGGTACAACGCATACTGCATGATTGGATCTG 1302
                ************* ************************************************

gene2CD         CTGTCAAATTCAGCACCATGATTGGCCTACACCAAAATGTGCCCTTGTCTCTGATGCCGG 1440
gene2endo       CTGTCAAATTCAGCACCATGATTGGCCTACACCAAAATGTGCCCCTATCTCTGATGCCGG 1362
                ******************************************* * *************

gene2CD         ATCGCGAGAAGCAAGAACACAGAAAGAGGATATGGTGGTCGGTGTATACTCTTGATCGCT 1500
gene2endo       ACCGCGAGAAACAAGAACACAGAAAGAGGATATGGTGGTCGGTGTATACTCTTGACCGCT 1422
                * ******** ****************************************** ****

gene2CD         TCTGGGGGGCTCAGATCGGCCAACCAGTTTCCATCAGAAACGAGGACATCGACGTCGATC 1560
gene2endo       TCTGGGGAGCTCAGATCGGTCAGCCAGTTTCTATCAGGGACGAGGACATCGACGTAGACC 1482
                ******* *********** ** ******** *****  ***************** ** *

gene2CD         CGCCTTCCATCGAGGGATTATCTCCCGAAAGCGCGGCTGAAGACTTTGCAGACGCCGAGT 1620
gene2endo       CGCCTTCCATCGAGGGGTTATCTCCTGAAAGTGCGGCTGAGGACTTTGCGGACGCTGAGT 1542
                **************** ******** ***** ******** ******** ***** ****

gene2CD         ATCTCACAGCCAACCACCGGCTGACCAATCTCGCCGCTCAAATTGCGTCGTTGATCTACA 1680
gene2endo       ATCTGACAGCCAACCACAGGTTGACTAATCTTGCTGCTCAGATTGCGTCGTTGATCTACA 1602
                **** ************ ** **** ***** ** ***** ******************

gene2CD         GCCGGAAGAATCAGCGCACCTCCTTCTCTAGTAGGGTTCAACAAGCGCTGAGAGACTTGA 1740
gene2endo       GCCGGAAGAATCAGCGCACCTCCTTCTCGAGTAGAGTACAACAAGCGCTGAGAGACCTAA 1662
                **************************** ***** ** ***************** * *

gene2CD         CTAGCTGGCTACAAGGCCTGCCAGACTCTTTGCGAGCTGCTATGGAAGAACTTCCCCCTA 1800
gene2endo       CTAGCTGGCTACAAGCCCTGCCGGACTCTCTCCGAGCCGCCATGGAGGAGCTTCCCCCTA 1722
                *************** ****** ****** * ***** ** ***** ** **********

gene2CD         ACGCGACAGCGCCCATCACCGCTTTGCACCTCTCTTTTAACCAGGTCAGTCGAGTGGCGC 1860
gene2endo       ACGCGACGGCACCGATAACAGCCTTGCACCTCTCTTTCAACCAGGTCAGTAGGGTCACAC 1782
                ******* ** ** ** ** ** ************** ************ * **  * *

gene2CD         ATGTCTACTGTCCAACACGCTTC[TAACTCACGTAGTGTCTAATACGGGCGGCGAGACCAA] 1920
gene2endo       ATACCCACCGTTCAACGCGCTTC[TAACACAACTAGTGTCTAATACGAGCAGCAAGACCAA] 1842
                **  * ** ** **** ********** **  ************** ** ** *******

gene2CD         [TTGTGCTGTACGTCTTTCGC]ATGCGCCGAGAGGCTCGCAAGTCTATCGAGGAAGAGCGCC 1980
gene2endo       [TTGTGCTGTATGTTTTTCGT]ATGCACCGAGAGGCTCGCAAGTCAATTGAGGATGACCACC 1902
                ********** ** ***** **** ****************** ** ***** ** * **

gene2CD         CTCCTATTGGCGAAGTTGCTTTGACTCTTTCGGATGCCTGCATCCAATGTGCCCGACGCT 2040
gene2endo       CTCCCATTGGCGAAGTTGCTTTGACTCTTTCGGATGCCTGCATTCAATGTGCTCGACGCT 1962
                **** ************************************ ******** *******
```

```
gene2CD         CCTGTCGACTTTTAGTAGAGTCTTGGATCAATGGGTCCTTTCCCACGTTCGATGTTTCCT 2100
gene2endo       CTTGTCGACTCCTTGTAGAGTCTTGGATCAACGGATCCTTCCCAACTTTCGATGTTTCCT 2022
                * ********  * ***************** ** ***** ** ** *************

gene2CD         ATGTTCACCACCTCTTCTCATCATCCATCGTCCTCGCCATATCCAGTCTATCTCAAACCA 2160
gene2endo       ACGTTCACCACCTATTCTCGTCGTCCATCGTCCTCGCCATATCGAGTCTATCTCAGACAA 2082
                * ************ ***** ** ******************** ************ ** *
gene2CD         ACGAGTCTCAAAGCGACAGTGATGACTTTGATGCTGCGATGCAAATACTCAAACAACTAG 2220
gene2endo       ATGAGTCTCAAAGCGATAGTGACGACTTTGATGCTGCGATGCAAATACTCAAGCAGTTAG 2142
                * ************** ***** ***************************** **  ***

gene2CD         ACGAGAGTGGCAATTTCGCAGCGAGAGAGTTCTTGAAGAGCATGGAAGCCACCAGGGCTG 2280
gene2endo       ACGAGAGTGGCAATTTCGCGGCGAGAGAGTTCTTGAAGAGCATGGAAGCTACCAGAGCGG 2202
                ******************* ***************************** ***** ** *

gene2CD         CCCTAGACAGCATCGCTGCAGAAAGAAACCAAGCAGGTCAGGATAATCGAGAGCCTGATT 2340
gene2endo       CCCTTGACAGTATAGCTGCTGAGCGAAATCAGTCAGGACAGGACAATCGAGAGACTGATT 2262
                **** ***** ** ***** **  **** **  **** ***** ********* ******

gene2CD         CAGTGGCCATGCGTGGCGGGCCATTCTCTTTCCACGACCCGAGGATATTCGATGCACCCA 2400
gene2endo       CAATGGCCATGCGTGGCGGGCCATTCTCTTTCCACGACCCGAGAATGTTCGATGCACCCA 2322
                ** ************************************** ** *************

gene2CD         CGGACACGGCTAGGATGGCCCTGACGGAGCCTTCGTTCCAGGACTTGCTGTCCCAGTCTG 2460
gene2endo       CGGACACAGCTAGGATGGCCCTGACAGAGCCTTCGTTTCAAGACTTGTTGTCCCAGTCTG 2382
                ******* ***************** *********** ** ****** ************

gene2CD         ATCTGGATCTTCAGTTTCTTGAGTCATCTAATAATGATCAGGACTTCCCGGGATTCTTCT 2520
gene2endo       ATTTGGATCTTCAGTTCCTTGAGTCATCCAATAATGATCAAGACTTTCCGGGATTTTTCT 2442
                ** ************* *********** *********** ***** ******** ****

gene2CD         GGCCTAATGAGGGATTTCAAGGATGGATGAA 2551
gene2endo       GGCCTAATGAGGGATTCCAAGGA-------- 2465
                *************** ******
```

Figure C.6. Clustal alignment of ~2500 bp region encompassing *HUT2* on the CD chromosome (Chromosome 14, scaffold 24) and normal chromosome (Nh85516 on Chromosome 10), showing 90% identity at the nucleic acid level. The two putative introns are in bold.

not shown); and the putative ~810 amino acids encoded by gene 2 on the CD chromosome and normal chromosome showed 82% identity at the amino acid level (Fig. C.7). Using the NCBI BLAST search and the ClustalW2 sequence alignment program, the putative protein encoded by gene 2 on the CD chromosome was found to show highest identity with putative PUT3-like fungal specific transcription factors of *Aspergillus fumigatus* (41% identity) and *Aspergillus niger* (32% identity), hypothetical proteins of *Gibberella zeae* (*Fusarium graminearum*) (36% identity), *Magnaporthe grisea* (34% identity), and *Podospora anserine* (32% identity), and a putative fungal specific transcription factor of *Neosartorya fischeri* (31% identity). Gene 2 has a Zn_2Cys_6 binuclear cluster DNA-binding domain similar to that of transcription regulators like GAL4 and PUT3 (Todd and Andrianopoulos 1997, Sellick and Reece 2005).

Gene 3 had no putative introns. Clustal alignment of ~1600 bp region encompassing gene 3 on the CD chromosome (Chromosome 14, scaffold 24) and normal chromosome (Chromosome 4, scaffold 19), showed 75% identity at the nucleic acid level (Fig. C.8); the open reading frame of gene 3 on the CD chromosome and normal chromosome showed 82% identity at the nucleic acid level (data not shown); and the putative ~470 and ~533 amino acids encoded by gene 3 on the CD chromosome and normal chromosome, respectively, showed 87% identity at the amino acid level (Fig. C.9). Using the NCBI BLAST search and the ClustalW2 sequence alignment program, the putative protein encoded by gene 3 on the CD chromosome was found to show highest identity with hypothetical proteins of *Aspergillus oryzae* (73%) and *Aspergillus nidulans* (65%), and a putative β-aspartate-semialdehyde dehydrogenase (succinate-

```
gene2CDAA       MGADPSYFSHPPQLGLPLVLAEYG--LEPHHVFQLDPSSQLSDPSGHIRSINPYCDPSLL 58
Gene2endoAA     MLITSSNLIHHLRSRILLAISDQLTLIVIHHCYNIHVMSETPEPEGQRLRRS-------- 52
                *   .* : *  :  : *.:::    :  ** :::.  *: .:*.*:    .

gene2CDAA       LYPCLKHQSLRASDFGVLSESQGQRRRTHALRMSTTSCRFPMKWMFTNFFSCRKCHARKV 118
Gene2endoAA     -FGASRATPKNARIAYVHDEVPVSN--EMDLYMSTTRYRFPMKWISTNSFSCRKCHARKV 109
                 : . :  . .*    * .*   ..     * ****  ******: ** ***********

gene2CDAA       KCSGGTPCANCLQANIGAECSYPRRIRRVKVDQRYGSKVIVLWDNYM----LLEKRFIPP 174
Gene2endoAA     KCSGGTPCANCLQANIGAECSYPRRIRRVKVDQRYESKSSVLMDELLDEIHSLRKAVHTP 169
                ********************************** **  ** *: :     *.* . .*

gene2CDAA       EVANSTSSRRHETRALSFVSDGDAVNQESPAASVINADTGALQANPEATVVVDQTRPAPE 234
Gene2endoAA     EAANSTPSRRHETRAQSFVSEGDAVNQESPAASVINADTGILQANPEATVVVDQTRPAPE 229
                *.****.******** ****:****************** ******************

gene2CDAA       TDDSIRNPILEDRPWFFSLTPEMPMLIEEAADAPFATRFRQELSGKSQRHIPRTENVTDE 294
Gene2endoAA     TDDSIRNPILEDRPWFFSLTPEMPMLIEEAADAPFATRFRQELSGKSQRHIPRTENVTDE 289
                ************************************************************

gene2CDAA       ALTSSWNTPCPWPPASRARFLLKVALNTVCKRYYLVRRSATQRLLEQAIHNPDMCDLVSE 354
Gene2endoAA     ALTSSWNTPCPWPPASRARFLLKVALNTVCKRYYLVRRSATQRLLEQVIHNPDMCDLVSE 349
                ********************************************.***********

gene2CDAA       YKLFVLFALGEVYSTRTSPTKDKNQLPGISYYVRASRFLRVLTEQPRIDCVEVILMLVRL 414
Gene2endoAA     CKLFVLFALGEVYSTRTSPTKDKNQLPGIAYYIRASRFLRVLTEQPRIDCVEVILMLVRL 409
                 ***************************:**:**************************

gene2CDAA       LLLSGLQC----------MNRRYNAYCMIGSAVKFSTMIGLHQNVPLSLMPDREKQEHRK 464
Gene2endoAA     LVLSVCNANTRKSLYSLAMNRRYNAYCMIGSAVKFSTMIGLHQNVPLSLMPDREKQEHRK 469
                *:**  :.          ****************************************

gene2CDAA       RIWWSVYTLDRFWGAQIGQPVSIRNEDIDVDPPSIEGLSPESAAEDFADAEYLTANHRLT 524
Gene2endoAA     RIWWSVYTLDRFWGAQIGQPVSIRDEDIDVDPPSIEGLSPESAAEDFADAEYLTANHRLT 529
                ************************:**********************************

gene2CDAA       NLAAQIASLIYSRKNQRTSFSSRVQQALRDLTSWLQGLPDSLRAAMEELPPNATAPITAL 584
Gene2endoAA     NLAAQIASLIYSRKNQRTSFSSRVQQALRDLTSWLQALPDSLRAAMEELPPNATAPITAL 589
                ************************************.**********************

gene2CDAA       HLSFNQVSRVAHVYCPTRFMRREARKSIEEERPPIGEVALTLSDACIQCARRSCRLLVES 644
Gene2endoAA     HLSFNQVSRVTHTHRSTRFMHREARKSIEDDHPPIGEVALTLSDACIQCARRSCRLLVES 649
                **********:*.: .****:********:::*****************************

gene2CDAA       WINGSFPTFDVSYVHHLFSSSIVLAISSLSQTNESQSDSDDFDAAMQILKQLDESGNFAA 704
Gene2endoAA     WINGSFPTFDVSYVHHLFSSSIVLAISSLSQTNESQSDSDDFDAAMQILKQLDESGNFAA 709
                ************************************************************

gene2CDAA       REFLKSMEATRAALDSIAAERNQAGQDNREPDSVAMRGGPFSFHDPRIFDAPTDTARMAL 764
Gene2endoAA     REFLKSMEATRAALDSIAAERNQSGQDNRETDSMAMRGGPFSFHDPRMFDAPTDTARMAL 769
                ************************:******.**:*************:************

gene2CDAA       TEPSFQDLLSQSDLDLQFLESSNNDQDFPGFFWPNEGFQGWMNG 808
Gene2endoAA     TEPSFQDLLSQSDLDLQFLESSNNDQDFPGFFWPNEGFQG---- 809
                ************************************
```

Figure C.7. Clustal alignment of putative ~810 amino acids encoded by HUT2 on the CD chromosome (Chromosome 14, scaffold 24) and normal chromosome (Nh85516 on Chromosome 10), showing 82% identity at the amino acid level.

```
gene3CD   ------------------------------------------------------------
gene3endo ATAATAATTAGGACTTTCCGGGATTTTTCTGGCCAAATGAGGGATTTCAAGGATGGATGA 60

gene3CD   ------------------------------------------------------------
gene3endo ACGGCTGATGTGATACCTGGATACAAGTCTTGAAAGCGATTGAATTCTACTCTTCTCTTT 120

gene3CD   ------------------------------------------------------------
gene3endo ACATTTTACCTGACCTTCCCCCCTTGCTGAAGCCTGCTCAAGGTGTAGGAGAACCATCAA 180

gene3CD   ------------------------------------------------------------
gene3endo CTGTGAGAGTCCCGGTTTTGGAGATGAGATACTCTTCAATGGCAACGTCCTTTCCGGCCT 240

gene3CD   ------------------------------------------------------------
gene3endo CCTTACCATAGCCTGAAGACTTGATACCGCCGAAAGGAGACTCGGCACATGAAGCATTTC 300

gene3CD   ----------------------------------CATGAAAAGATGCTTACCGGTATT 24
gene3endo CTTGACATTTGTTAGCGTTGGCATCTCTTGCAGTTACATGACAATATACTTACCTGTATT 360
                                             ***** ** ** ****** *****

gene3CD   CATTCCAATCATACCAGCCTCAAGGCTCTCGAGAAGCCTCCATGTACGACTGGCATCCTT 84
gene3endo CATTCCAATCATACCAGCCTCAAGGCTCTCGAGAAGCCTCCACGTACGACTGACGTCTTT 420
          ****************************************** ********* * ** **

gene3CD   GGTGTAAAAGTATGAAGCCAAGCCCATAGATGTGTTGTTGGCCAACTCAATAGCTTCCTC 144
gene3endo GGTGTAAAAGTACGAAGCCAGGCCCATAGATGTGTTGTTGGCGAACTCCACAGCTTCTTC 480
          ************ ******* ******************** ***** * ****** **

gene3CD   CTCTGTCTCGAATTTGTAGAGGCCTAGTAGAGGACCAAAGATCTCCTCTTGCGTCGTCAA 204
gene3endo CTCCGTCTCGAACTTGTAGATGCCCAGGAGAGGACCGAAGATCTCCTCTTGTGTCGTCAA 540
          *** ******** ******* *** ** ******** ************** ********

gene3CD   CATGTCGGGCGTCATTCCTGAGATGATGGTAGGTTCAAAGAAGTAGCCGTCCTTGAGGTC 264
gene3endo CATATTAGGCGTCATTCCCGAGATGATGGTCGGTTCAAAGAAGTAGCCGTTCTTTAGGTC 600
          *** *  ************ ************ ******************** *** *****

gene3CD   AGCTGGTCTCTTTCCGCCGCATAGGACCTTGCCTCCTTTGCTAACAGCATCGGAAACATG 324
gene3endo AGCAGGCCTCTTTCCGCCACATAGAATCTTGCCTCCTTTGCTAACGGCATCGGATACATG 660
          *** ** ************ ***** * ****************** ******** *****

gene3CD   CTTCTCAACTTTTTCGACTTGGCGGCCTGTTGTTAGGGGACCCATGGTCGTCCCTGTTTC 384
gene3endo CTTCTCGACCTTTTCAACTTGTCGGCCTGTTGTCAGGGGACCCATGGTCGTCCCTGTCTC 720
          ****** ** ***** ***** ************ ********************** **

gene3CD   GGCGCCATGGCCTACCTTCAGCTTTTGCGTGGCTTCCAACATCTTTTGGGTAAACTTGTC 444
gene3endo GGCGCCATGGCCCACTTTGAGCTTTTGCGTGGCCTCTAACATCTTTTGGGTAAACTTGTC 780
          ************ ** ** ************** ** **********************

gene3CD   GTACACGCCGCTTTGCACGTAAACTCGGTTGGCATGCGTGCAAGCCTGACCAGCTGTGCG 504
gene3endo GTACACGCCGCTTTGCACGTAAACTCGGTTGGCGTGCGTGCAAGCCTGACCAGCTGTGCG 840
          ********************************** *************************

gene3CD   CCACTTGAGGATCATGAGCGCGGCGACAGCTTGATCTAGGTCGCCGTCGTCAAAGATGAG 564
gene3endo CCACTTGAGGATCATAAGTGCGGCAACAGCTTGGTCTAGGTCACCATCGTCAAAGATGAG 900
          *************** ** ***** ******** ******** ** **************

gene3CD   GAACGGACAATTGCCGCCAAGTTCCATCGTGACCTTCTTCAGACCCTCGGCGCAATGCTT 624
gene3endo GAACGGACAGTTGCCGCCAAGTTCCATGGTCACCTTCTTCAGACCCTCGGCGCAATGCTT 960
          ********* ***************** ** ******************************

gene3CD   GGCGACAATGCTTCCGATATTGGTGCTTCCTGTAAAGGTGACCTTTCGGACCAAGGGATG 684
gene3endo GGCGACAATGCTGCCCACGTTGGTGCTTCCTGTGAAGGTGACCTTTCGCACCAAGGGATG 1020
          ************ ** *  ************** ************** ************

gene3CD   CTTGCAGAGCCTTTCGCTGACCGATGGCGTGTTGGTGTTGTCGGTTGAGATGACGTTAAG 744
gene3endo CTTGCAGAGCCTTTCGCTGACCGATGGTGTGTTGGCGTTGTCTGTCGAGATGACGTTGAG 1080
          *************************** ******* ****** ** ********** **
```

```
gene3CD   CACACCAGCGGGCAGTCCAGCTCGGAGGGCGAGATCGGCGAGGGCCATGACACTGAAGGG 804
gene3endo CACACCGGCGGGCAGTCCAGCTCGGAGAGCCAGATCAGCGAGGGCCATGACGCTGAAGGG 1140
          ****** ******************** ** ***** ************** ********

gene3CD   ACTCTCGGGAGAAGGCTTGACAACCATGGTGCAACCGGCAGCTAAAGCCGCAGCAACCTT 864
gene3endo ACTCTCGGGAGAAGGCTTAACAACCATGGTGCAGCCGGCAGCCAAAGCCGCAGCGACCTT 1200
          ****************** ************** ******** *********** *****

gene3CD   GCGGATAATCATAGCAACAGGGAAGTTCCAAGGCACAAGAGCGACGCTCACACCAATAGG 924
gene3endo GCGTATAATCATAGCAACAGGAAAGTTCCAAGGCACAAGAGCGACGCTCACACCAATGGG 1260
          *** ***************** ********************************** **

gene3CD   TTGCTTAATAACAAACGTCCGCCGGTTAGACACAGACGGTAGAGCAATTGTCCCTCGAAC 984
gene3endo TTGCTTAATAACAAACGTTCGCCGGTTAGACACAGAAGGCAGGGCAATTGTACCGCGAAC 1320
          ****************** ***************** ** ** ******** ** *****

gene3CD   GCGCTCAGCTTCGCCAGCGAACCACCAAGCGAAGCCAAGAGCATAATCGACTTCACCTAT 1044
gene3endo ACGCTCAGCTTCGCCAGCGAACCACCATGCGAAGCCAAGAGCATAATCGACTTCGCCCAC 1380
           ************************* ************************** ** *

gene3CD   GGCCTCGGTCATGGGCTTTCCCGTTTCGTAGACGACGATCTTGGCAATGTCCTCGCGGGC 1104
gene3endo GGCCTCGGCCATGGGCTTTCCCGTCTCATAGACGACAATCTTGGCAATGTCCTCGCGGGC 1440
          ******** *************** ** ******** ************************

gene3CD   GTTGGTGATGAGCTCGTGCCATTTCAGGAGAATCTTGGCTCGTTCGCGGGGGTTGGTGTA 1164
gene3endo GTTGGTGATTAGCTCGTGCCATTTGAGGAGGATCTTGGCTCGTTCGCGGGGATTGGTGTA 1500
          ********* ************** ***** ******************** ********

gene3CD   GCGGAAGCTGTTGAATGCAGTTTGCGAGGATTCGATGTATTGGTCGACGTCTTCGACTTG 1224
gene3endo GCGGAAGCTGTTGAAGGCGGTCTGAGATGACTCGATGTATTGGTCGACGTCTTCGACTTG 1560
          *************** ** ** ** ** ** ****************************

gene3CD   GTTGGTGGGCGAGGTGGCCCAGATCTTGCCAGAGCCGGGATCTACACATATTAGAAAATA 1284
gene3endo GTTGGTGGGCGAGGTGGCCCAGATCTTGCCAGAGCCGGGATCTATAAATAT--------- 1611
          ****************************************** * ****

gene3CD   TCAATATGTTAAGTTGAATGTTTGAGAACTCACCCTCGACATTAAAGCGCTTGCCGGCCT 1344
gene3endo ------------------------------------------------------------

gene3CD   GGGCTTGCACCCATTTGCCATTGAGCAGCGATGCCTCATGGAGGAGGGAGGGGTCGTTCA 1404
gene3endo ------------------------------------------------------------

gene3CD   ACTTGGAGATTGGTCAGTGAGGGGTAGACTTCATGAATGAGGGGGAGGGGTAGTTACTGT 1464
gene3endo ------------------------------------------------------------

gene3CD   GAAGGGGAGAGACATTGTGCTTTGTTGTGTAAGTAGATTGGCTGAAGAGTCTGTGATTCT 1524
gene3endo ------------------------------------------------------------

gene3CD   TGTCCGTCAACCGACATGATGAAACTGAGAT 1555
gene3endo ------------------------------
```

Figure C.8. Clustal alignment of ~1600 bp region encompassing gene 3 on the CD chromosome (Chromosome 14, scaffold 24) and normal chromosome (Nh79751 on Chromosome 4), showing 75% identity at the nucleic acid level.

```
gene3CDAA       MSVSSCRLTDKNHRLFSQSTYTTKHNVSPLHSNYPSPSFMKSTPHMCVDPGSGKIWATSP 60
gene3endoAA     -------------------------------------------MFIDPGSGKIWATSP 15
                                                           * :************

gene3CDAA       TNQVEDVDQYIESSQTAFNSFRYTNPRERAKILLKWHELITNAREDIAKIVVYETGKPMT 120
gene3endoAA     TNQVEDVDQYIESSQTAFNSFRYTNPRERAKILLKWHELITNAREDIAKIVVYETGKPMA 75
                ************************************************************:

gene3CDAA       EAIGEVDYALGFAWWFAGEAERVRGTIALPSVSNRRTFVIKQPIGVSVALVPWNFPVAMI 180
gene3endoAA     EAVGEVDYALGFAWWFAGEAERVRGTIALPSVSNRRTFVIKQPIGVSVALVPWNFPVAMI 135
                **:************************************************************

gene3CDAA       IRKVAAALAAGCTMVVKPSPESPFSVMALADLALRAGLPAGVLNVISTDNTNTPSVSERL 240
gene3endoAA     IRKVAAALAAGCTMVVKPSPESPFSVMALADLALRAGLPAGVLNVISTDNANTPSVSERL 195
                ************************************************:*********

gene3CDAA       CKHPLVRKVTFTGSTNIGSIVAKHCAEGLKKVTMELGGNCPFLIFDDGDLDQAVAALMIL 300
gene3endoAA     CKHPLVRKVTFTGSTNVGSIVAKHCAEGLKKVTMELGGNCPFLIFDDGDLDQAVAALMIL 255
                ****************:*******************************************

gene3CDAA       KWRTAGQACTHANRVYVQSGVYDKFTQKMLEATQKLKVGHGAETGTTMGPLTTGRQVEKV 360
gene3endoAA     KWRTAGQACTHANRVYVQSGVYDKFTQKMLEATQKLKVGHGAETGTTMGPLTTGRQVEKV 315
                ************************************************************

gene3CDAA       EKHVSDAVSKGGKVLCGGKRPADLKDGYFFEPTIISGMTPDMLTTQEEIFGPLLGLYKFE 420
gene3endoAA     EKHVSDAVSKGGKILCGGKRPADLKNGYFFEPTIISGMTPNMLTTQEEIFGPLLGIYKFE 375
                *************:***********:**************:**************:****

gene3CDAA       TEEEAIELANNTSMGLASYFYTKDASRTWRLLESLEAGMIGMNTGKHLFM---------- 470
gene3endoAA     TEEEAVEFANNTSMGLASYFYTKDVSRTWRLLESLEAGMIGMNTGKYIVMMPTLTNVKEM 435
                *****:*:****************.*********************::.*

gene3CDAA       ------------------------------------------------------------
gene3endoAA     LHVPSLLSAVSSLQAMVRRPERTLPLKSISSPKPGLSQLMVLLHLEQASARGEGQVKCKE 495


gene3CDAA       -------------------------------------
gene3endoAA     KSRIQSLSRLVSRYHISRSSILEIPHLARKIPESPNYY 533
```

Figure C.9. Clustal alignment of putative ~470 and ~530 amino acids encoded by gene 3 on the CD chromosome (Chromosome 14, scaffold 24) and normal chromosome (Nh79751 on Chromosome 4), respectively, showing 87% identity at the amino acid level.

semialdehyde dehydrogenase) of *Pyrenophora tritici-repentis* (60%), *Aspergillus fumigatus* (55%), and *Coccidioides immitis* (55%).

Gene 4 had no putative introns. Clustal alignment of ~1600 bp region encompassing gene 4 on the CD chromosome (Chromosome 14, scaffold 24) and normal chromosome (Chromosome 4, scaffold 19), showed 86% identity at the nucleic acid level (Fig. C.10); the open reading frame of gene 4 on the CD chromosome and normal chromosome showed 88% identity at the nucleic acid level (data not shown); and the putative ~423 and ~508 amino acids encoded by gene 4 on the CD chromosome and chromosome 4, respectively, showed 93% identity at the amino acid level (Fig. C.11). Clustal alignment of the putative amino acids encoded by gene 4 on the CD chromosome and by another putative homolog on chromosome 2 showed only 68% identity. Using the NCBI BLAST search and the ClustalW2 sequence alignment program, the putative protein encoded by gene 4 was found to show highest identity with a putative bifunctional aspartokinase/HS dehydrogenase of *Pyrenophora tritici-repentis* (65%), a HS dehydrogenase of *Neurospora crassa* (64%) *and Pyrenophora tritici-repentis* (59%), and hypothetical proteins of *Aspergillus oryzae* (67%), *Magnaporthe grisea* (66%), *Gibberella zeae* (*Fusarium graminearum*) (66%), *Botryotinia fuckeliana* (65%), *Sclerotinia sclerotiorum* (65%), and *Chaetomium globosum* (63%).

Gene 5 had no putative introns. Clustal alignment of the ~910bp region encompassing gene 5 on the CD chromosome (Chromosome 14, scaffold 24) and normal chromosome (Chromosome 4, scaffold 19), showed 90% identity at the nucleic acid level (Fig. C.12); the open reading frame of gene 5 on the CD chromosome and normal

```
gene4CD         ------------------------------------------------------------
gene4endo       ATGATGAGGGTGAGGTGCAGGGACTTATATGCCACTTGTTCCGGCTCGGAGATGTTCATA 60


gene4CD         ---------------------------------------------ATCTTGACAAAT 12
gene4endo       CTTCTCGACCTCCAAACATCTAAAACCGAGTTTTGTGTTACATCTCGAAGCTTTACAAAT 120
                                                            * *** ******


gene4CD         TGTTCCGCGGATGTTCCACCCGTTAGGGGCGACCTAATTGTTGCTGACGCGGAGAGTGTC 72
gene4endo       CGTTCTGCGGATGTTCCACCCGTTGTGGGCAACCTAATTATTGTTGCCGCGGATAGTGTC 180
                 **** ******************  **** ******** *** ** ****** ******


gene4CD         TTGTCAGCATCTGATTATGTTAACGAACTCGTACATCGTTTCGGCCTCTACACCTTCTAT 132
gene4endo       TCGTCAGCATCTGATTATGTCAACGAACTCGTGAACCGTTTCAGCATCCACTCCGTCCGT 240
                * ****************** ***********  * ****** ** ** ** ** **  *


gene4CD         AGCCGAAGTGTTGACGAAGGGAAACTGTAGTAACATGATCTTGATTATCGCCTTTTAAAT 192
gene4endo       AGCCGGAGAGCTTACGAAGGGAATCTGTAGTAATACGATCTTTATTATCGCCTTTTCAAT 300
                ***** ** * * ********** ********* * ****** ************* ***


gene4CD         AAAATGCTTCAAGTTAATCATGATCTGGTTATGTTACGCGGCTCACGAGGACGGCGCGTC 252
gene4endo       GAAAAGCTTCAATTTAATCATGATCTGGTTATGTTACGCTGCTCACGAGGACGGCGCGTC 360
                 *** ******* ************************** ********************


gene4CD         GCACTGACAAGCCGAATCCGCTCCGAGACCGTTGGCACCCGATTTGAGATTCGGAGATTC 312
gene4endo       ATACTGACAAGCCGAATCCTTTCCGAGACGGTCGGTGCTCGAGTTGGGATCCGGAGATTT 420
                  *****************  ******** ** **  * *** *** *** ********


gene4CD         CAAGAACATGGACTTTTTCATTCTTAAAAGACAAGAAACCTCCTGCCATTCCGGATCTGC 372
gene4endo       CAAGTACACGGCTTGATTCATCCTTAAAACGCATGGTCTATGCTGTCCTTCTAGATCTCC 480
                **** *** **  *  ***** *******  ** *     * *** * ***  ***** *


gene4CD         AGCTGAGTTTGAGACTCACGGCCTATCACAAGGAGACTTCAGTCATCCGCCCTCAGCCTC 432
gene4endo       AGCCTGGTTTGGGACTCCGAATCCATCACAAGAAGACT----------ACTCTCA---TC 527
                ***   ***** *****     * ******** *****           * ****   **


gene4CD         CCAATATGGCAACGTCCACCCATGAGGTTTCCATTGCCGTGATCGGTGAGTAGATACCTT 492
gene4endo       CCAACATGACGCGCTCTATCCCGGAGGTGTCCATCGCCGTGATCGGTGAGTAGATGCCGT 587
                **** *** *    ** * **  ***** ***** ******************** ** *


gene4CD         CAAGGGCTACTATTGCCTATAATCAGCCAATAACACTTGAAATAGGTGCCGGCGGCATTG 552
gene4endo       CAAGGACTACTATTGCTTAGAATCAGCCACTAACACTTGAAATAGGCGCTGGCGGCGTTG 647
                ***** ********** ** ********* *************** ** ****** ***


gene4CD         GATCCGTCTTCCTCCAGCAACTCGCCTGGGTGGCCAAGAACAAGACCTCGCACCGTCTGA 612
gene4endo       GATCCGTCTTCCTCCAGCAACTCGCTTGGGTAGCCAAGAACAAGACCTCGCACAGTCTGA 707
                ************************* ***** ******************** ******


gene4CD         GGCTCGTCTACGTCGCCATCATCGACAAGGCCCTCTACCACGCCGATTATGCATCCATCG 672
gene4endo       GACTCGTTTACGTCGCCATCATCGACAAGGCTCTCTACCACGCCGATTATGCATCTATCG 767
                * ***** ********************** ********************** ****


gene4CD         AAATTGCCTCTGCAGTGCCGACGCTCGAAGAAAAGGGCGGACCACTACCTACGGTCCCCC 732
gene4endo       ATATTGTCTCTGCTGTGCCGACGCTCGAAGAAAAGGGCGGACCACTACCTACAATCTCCC 827
                * **** ****** ************************************  ** ***


gene4CD         AAACCATCGAGTATCTGGCAAATGCACCTGGCAAGGTTATTGTCGTCGACAACACCAGCA 792
gene4endo       AAACCATCGAGTATCTGACAGGTGCACCTGGCAAGGTTATTGTCGTCGACAACACCAGCA 887
                ***************** **  ************************************


gene4CD         GCCAGGCAGTCGCCGAGGCGTATCCTTCGTTTCTTGCTAGGGGTTTCAGCATCGTCACTC 852
gene4endo       GCCAGGCAGTCGCAGAGGCGTATCCTTCGTTTCTTTCCAGGGGCTTTTGTGTCGTTACTC 947
                ************* ******************** * ***** **  *  **** ****


gene4CD         CTAACAAGAAGGCTTTCTCTGGAAGCTGGAAGTTGTGGCAAGACATCTTTGCTGCTGAGG 912
gene4endo       CCAACAAGAAGGCCTTCTCTGGAAGCTGGAAGTTGTGGGAAGACATCTTTGCTGCTGAGG 1007
                * ************ ************************ ********************
```

```
gene4CD         GCACGGCCGGTTCCATGGTGTACCACGAGTGCTCGGTTGGCGCCGCTTTGCCGATAATCT 972
gene4endo       GCACTGCCGGTTCCATGGTGTACCACGAGTGCTCAGTTGGCGCCGCTTTGCCTATCATCT 1067
                **** *************************** ***************** ** ****

gene4CD         CGACATTGAAGGAGCTCATAGCGACCGGTGATGAGATCACTCGAATTGAGGGTGTGTTTA 1032
gene4endo       CGACATTGAAGGAGCTCATCGCGACCGGCGATGAGATCACTCGAATTGAGGGTGTGTTTA 1127
                ****************** ******** ******************************

gene4CD         GCGGCACAATGTCATATCTCTTCAACAACTTTGCCCCTACTCAGGGAACCGGCGGGAAGT 1092
gene4endo       GCGGCACAATGTCATATCTGTTCAACAATTTTGCCCCTACTCAGGGAACCGGAGGGAAGT 1187
                ****************** ******** ********************** *******

gene4CD         GGTCGGACGAGGTCAAGAGGGCAAAGCAATTGGGGTACACTGAGCCGGATCCTCGTGATG 1152
gene4endo       GGTCGGACGAAGTCAAGAGAGCGAAGCAATTGGGATACACCGAGCCTGATCCTCGCGATG 1247
                ********** ******** ** ********** ***** ***** ******** ****

gene4CD         ACCTTAACGGTCTCGATGTCGCCCGCAAGATCACCATCCTCGCCCGGTTAGCTGGTCTTC 1212
gene4endo       ATCTCAATGGCCTTGACGTAGCCCGCAAGATCACCATCCTTGCCCGGTTAGCTGGTCTTC 1307
                * ** ** ** ** ** ** ******************** ******************

gene4CD         CCATTGAGTCCGCAACAGCTTTCCCCGTCCAGAGCCTTATCCCCAAAGAGCTAGAGAGTG 1272
gene4endo       CCATTGAGTTCGTAACAGCCTTCCCCGTCCAGAGCCTTATTCCCAAAGAGCTAGAGAGTG 1367
                ********* ** ****** ******************** ******************

gene4CD         TTAAGAGCGGCGACGAATTTCTTCAAAGACTTCCTGAGTTCGACTCACAGATGACTCAGC 1332
gene4endo       TCAAGAGCGGCGACGAATTCCTTCAAAGACTCCCCGAGTTTGACACACATATGAACGAGC 1427
                * ***************** ********** ** ***** *** **** ****   ***

gene4CD         ACAAGGAAGCTGCGGGAAAGGCCGGCAAGGTAGTGCGGTTCATCGGTTCCGTCGACGTTG 1392
gene4endo       ACAAGGAAGCTGCCAAAAAGGCCGACAAGGTGGTGCGGTTTATCGGTTCTGTGGACGTTG 1487
                *************   ******** ****** ******** ******** ** *******

gene4CD         CGACGAAGCAGCTCAAGGTTGGCCTCGAGGCTTTTGACATGTCACATCCCATTGCTTCAC 1452
gene4endo       TGACAAAGCAGCTAAAGGTTGGCCTGGAGGCCTTTGACAAGTCGCATCCCATTGCTTCAC 1547
                 *** ******** *********** ***** ******* *** ****************

gene4CD         TCCAAGGAAGTGACAACATCATCAGCTTCTATACTAAGCGATATGGTGATTTACCTTTGA 1512
gene4endo       TCAAGGGGAGTGACAACATCATCAGCTTCTATACTAGGCGATATGGTGATTTGCCCTTAA 1607
                ** * ** ***************************** *************** ** ** *

gene4CD         TTGTTCAGGGTGCTGGCGCAGGTGGTCCTGTTACGGCTATGGGCGTGTTGGGTGATCTTC 1572
gene4endo       TCGTTCAGGGTGCTGGTGCGGGTGGTCCTGTTACGGCTATGGGTGTTTTGGGCGATCTCC 1667
                * ************** ** ********************** ** ***** ***** *

gene4CD         TCAAGGTGCTTACAAGGATAGCTT 1596
gene4endo       TGAAGGTACTCACAAGGATAGCG- 1690
                * ***** ** ***********
```

Figure C.10. Clustal alignment of ~1600 bp region encompassing gene 4 on the CD chromosome (Chromosome 14, scaffold 24) and normal chromosome (Nh93154 on Chromosome 4), showing 86% identity at the nucleic acid level.

```
gene4CDAA       -----------------------------------MLTNCSADVPPVRGDLIVADAESV 24
gene4endoAA     MMRVRCRDLYATCSGSEMFILLDLQTSKTEFCVTSRSFTNRSADVPPVVGNLIIVAADSV 60
                                                  :** ******* *:**:. *:**

gene4CDAA       LSASDYVNELVHRFGLYTFYSRSVDEGKLMPSRATIAYNQP------------------- 65
gene4endoAA     SSASDYVNELVNRFSIHSVRSRRAYEGNLMLTRTARHTDKPNPFRDGRCSSWDPEISSTR 120
                 **********:**.::::. ** . **:** :*::   ::*

gene4CDAA       ----------------------------ITLEIGAGGIGSVFLQQLAWVAKNKTSHRL 95
gene4endoAA     LDSSLKRMVYAVLLDLQPGLGLRIHHKKTTLIPTMGAGGVGSVFLQQLAWVAKNKTSHSL 180
                                           :   :****:****************** *

gene4CDAA       RLVYVAIIDKALYHADYASIEIASAVPTLEEKGGPLPTVPQTIEYLANAPGKVIVVDNTS 155
gene4endoAA     RLVYVAIIDKALYHADYASIDIVSAVPTLEEKGGPLPTISQTIEYLTGAPGKVIVVDNTS 240
                ********************:*.***************:.******:.************

gene4CDAA       SQAVAEAYPSFLARGFSIVTPNKKAFSGSWKLWQDIFAAEGTAGSMVYHECSVGAALPII 215
gene4endoAA     SQAVAEAYPSFLSRGFCVVTPNKKAFSGSWKLWEDIFAAEGTAGSMVYHECSVGAALPII 300
                ************:***.:***************:*************************

gene4CDAA       STLKELIATGDEITRIEGVFSGTMSYLFNNFAPTQGTGGKWSDEVKRAKQLGYTEPDPRD 275
gene4endoAA     STLKELIATGDEITRIEGVFSGTMSYLFNNFAPTQGTGGKWSDEVKRAKQLGYTEPDPRD 360
                ************************************************************

gene4CDAA       DLNGLDVARKITILARLAGLPIESATAFPVQSLIPKELESVKSGDEFLQRLPEFDSQMTQ 335
gene4endoAA     DLNGLDVARKITILARLAGLPIEFVTAFPVQSLIPKELESVKSGDEFLQRLPEFDTHMNE 420
                ********************** .*****************************::*.:

gene4CDAA       HKEAAGKAGKVVRFIGSVDVATKQLKVGLEAFDMSHPIASLQGSDNIISFYTKRYGDLPL 395
gene4endoAA     HKEAAKKADKVVRFIGSVDVVTKQLKVGLEAFDKSHPIASLKGSDNIISFYTRRYGDLPL 480
                ***** **.***********.************ *******:**********:*******

gene4CDAA       IVQGAGAGGPVTAMGVLGDLLKVLTRIA 423
gene4endoAA     IVQGAGAGGPVTAMGVLGDLLKVLTRIA 508
                ****************************
```

Figure C.11. Clustal alignment of putative ~420 and ~500 amino acids encoded by gene 4 on the CD chromosome (Chromosome 14, scaffold 24) and normal chromosome (Nh93154 on Chromosome 4), respectively, showing 93% identity at the amino acid level.

```
Gene5CD          CATGGTTTGTCTATGAATCTTCCTTCAGAAGCGCCTCAAACGCTTGTGTAAAGTCCGCCT 60
Gene5endoRC      ------------TGAATCCTCCTTTAGAAGAGCTTCAAAGGCCTGTGTGAAATCCGCCT 47
                           ****** ***** ***** ** ***** ** ***** ** *******

Gene5CD          TCATATCTTCAAGGTCCTCAATACCGACACTAACACGAATGACAAGCGGATCCTCTCCCT 120
Gene5endoRC      TCATATCTTCAAGGTCTTCAACACCAACACTAACACGAATGACGAGAGGATCCTCTCCCT 107
                 ********************* **** *** ********************* ** **********

Gene5CD          TGCTACTCATGGCACGCCACTCAACCAAACTCTCAACTCCTCCCAAGCTTGCGGCGTGCT 180
Gene5endoRC      TGCTACTCATGGCACGCCACTCTACCAAACTCTCAACTCCTCCCAAGCTTGCAGCGTGCT 167
                 ********************** ****************************** *******

Gene5CD          GGAAGATGTATAGCTTACTGGCCAACCGCTTAGCATATTCGGCCTTCTTGAGCCACATGC 240
Gene5endoRC      GGAAGATGTATAACTTGCTGGCCAATCGCTTAGCGTATTCAGCCTTTTTGAGCCACATAC 227
                 ************ *** ******** ******** ***** ***** *********** *

Gene5CD          TGAACACGGGCCCGTAACCCCCGGGCATCTGCTTCTGCAGCCAGCCCTCCTCTAGAGCGT 300
Gene5endoRC      TGAACACGGGTCCGTAACCCCCGGGCATCTGCTTCTGCAGCCAGCCTTCCTCCAGGGCGT 287
                 ********** ********************************** ***** ** ****

Gene5CD          CCTTTTGGATCGAGGCATGCGACACCTTCAGAACCGCTCTTCCAACGACCGAGGTTGGGT 360
Gene5endoRC      CCTTTTGGATCGAGGCGTGTGACACCTTGAGAACTGCTCGTCCAATGACCGAGTTTGGGT 347
                 ***************** ** ******** ***** **** ***** ******* ******

Gene5CD          TCTGGATGCCATTATGAAGCCATGCGACAAGGCTCTCGGTCGTCTTTGCCTGCCGCGTTA 420
Gene5endoRC      CCTGAATGCCATCATGGAGCCATGCGACGAGGCCCTCAGTCGTCTTGGCCTGCCGCGTTA 407
                  *** ******* *** *********** **** *** ******** **************

Gene5CD          CGCGCAGATGGAGGGTACGCAGGGATCGGATACCCAACCACCCCTCCAAGCTGCCCATAA 480
Gene5endoRC      CGCGCAGATGGAGGGTACGCAGGGATCGGATGCCCAGCCATCCCTCCAAGCTGCCCATGA 467
                 ******************************** **** *** ***************** *

Gene5CD          CACTTCCCAAGACCAGACGCTCCTCCCGCAACGTCTCCTCCCATCCTTCCTTGACACGAT 540
Gene5endoRC      CACTTCCCAAGACCAGACGCTCCTCCCGCAGCGTCTCCTCCCATCCTTCCTTGACACGAT 527
                 ****************************** ******************************

Gene5CD          CAGGGTGGATGACAACGATGCCACAGAGCATATCCGAGTGACCACCGATATACTTGGTTC 600
Gene5endoRC      CGGGGTGGATGACGACGATACCACAGAGCATATCTGAGTGACCGCCGATATACTTGGTTC 587
                 * *********** ***** ************** ******** ****************

Gene5CD          CGCTGTGCATCACCATGTCGGCGCCAAATTGGAGGGGATCCTGCAGAGGAGGTGGACCAA 660
Gene5endoRC      CGCTGTGCATCACCATATCGGCACCAAACTGTAGAGGGTCCTGCAGAGGAGGGGGACCAA 647
                 ***************** ***** ***** ** ** ** ************** *******

Gene5CD          AAGTCGAGTCAACGGTAAGAATAGCTCCCACTGCACGAGCTCTCTCCTTATAATAAACAA 720
Gene5endoRC      AGGTCGAGTCAACGGTAAGAATAGCTCCCACTGCACGAGCTCTCTCCTTATAATAAGCAA 707
                 * ************************************************** ***

Gene5CD          GATTACGTGCCTCACCGGTCGGGTTAAGCGGCGTCTCGACGTGGAGCATGTCGCCTGGAC 780
Gene5endoRC      GGTTGCGAGCCTCGCCGGTCGGGTTAAGCGGTGTCTCGATGTGGAGCATGTCACCCGGGC 767
                 * ** ** ***** ***************** ******* ************ ** ** *

Gene5CD          CCATCTGGTCGATGTCGTCGAGGCTCAACTTCTTGAGGCCGTTGAGCTTGGAGATGACGT 840
Gene5endoRC      CCATCTGATCGATATCGTCAAGAGTCAACTTCTTCAAGCCATTGAGCTTAGAGATGACGT 827
                 ******* ***** ***** **  ********** * *** ******** **********

Gene5CD          CGATGACTCCATGGACCCCGTGGTATCCTTCCGTGAGAAAGATCTTTTTCGGGTTAACCA 900
Gene5endoRC      CGATAACTCCATGGACCCCGTGGTATCCTTCCGTGAGAAAGATCTTTTTGGGGTTCACCA 887
                 **** ****************************************** ***** ****

Gene5CD          AGACCAGCAT 910
Gene5endoRC      AAACCAGCAT 897
                 * ********
```

Figure C.12. Clustal alignment of ~910 bp region encompassing gene 5 on the CD chromosome (Chromosome 14, scaffold 24) and normal chromosome (Nh93153 on Chromosome 4), showing 90% identity at the nucleic acid level.

chromosome showed 90% identity at the nucleic acid level (data not shown); and the putative ~300 amino acids encoded by gene 5 on the CD chromosome and normal chromosome showed 96% identity at the amino acid level (Fig. C.13). Using the NCBI BLAST search and the ClustalW2 sequence alignment program, the putative protein encoded by gene 5 was found to show highest identity with cystathionine-β-lyase of *Aspergillus fumagatus* (63%), cystathionine-γ-synthase (O-succinyl-L-HS succinate-lyase) of *Neosartorya fischeri* (57%), *Pyrenophora tritici-repentis* (54%), and *Aspergillus clavatus* (50%), and hypothetical proteins of *Magnaporthe grisea* (58%) and other fungal species.

Pea rhizosphere competition assays

In the first competition assay isolate HT5 was paired with transformant HT5-2B07 in order to determine if HT5-2B07 has a competitive advantage due to HS utilization contributing to competitive ability. The second experiment paired transformant Tr78.2 with transformant HT5-2B07; since both of these isolates are HUT^{+} one would expect them to have the same degree of rhizosphere competitive ability unless there are other competitive genes on the region of the *PDA1* CD chromosome that was retained after transformation. The third experiment paired isolate HT5 with transformant HT5-2B07Δ3; if the HS gene cluster identified in this study is indeed responsible for HS utilization, and hence increased rhizosphere competence, then disruption of one of the HS genes (in this case, gene 3), should result in a loss of that increased competitive ability and neither HT5 nor HT5-2B07Δ3 should have an increased competitive ability over the other.

```
gene5CDAA       MLVLVNPKKIFLTEGYHGVHGVIDVISKLNGLKKLSLDDIDQMGPGDMLHVETPLNPTGE 60
gene5endoAA     MLVLVNPKKIFLTEGYHGVHGVIDVISKLNGLKKLTLDDIDQMGPGDMLHIETPLNPTGE 60
                **********************************:**************:*********

gene5CDAA       ARNLVYYKERARAVGAILTVDSTFGPPPLQDPLQFGADMVMHSGTKYIGGHSDMLCGIVV 120
gene5endoAA     ARNLAYYKERARAVGAILTVDSTFGPPPLQDPLQFGADMVMHSGTKYIGGHSDMLCGIVV 120
                ****.**************************************************** 

gene5CDAA       IHPDRVKEGWEETLREERLVLGSVMGSLEGWLGIRSLRTLHLRVTRQAKTTESLVAWLHN 180
gene5endoAA     IHPDRVKEGWEETLREERLVLGSVMGSLEGWLGIRSLRTLHLRVTRQAKTTEGLVAWLHD 180
                ****************************************************.******: 

gene5CDAA       GIQNPTSVVGRAVLKVSHASIQKDALEEGWLQKQMPGGYGPVFSMWLKKAEYAKRLASKL 240
gene5endoAA     GIQDPNSVIGRAVLKVSHASIQKDALEEGWLQKQMPGGYGPVFSMWLKKAEYAKRLASKL 240
                ***:*.**:************************************************** 

gene5CDAA       YIFQHAASLGGVESLVEWRAMSSKGEDPLVIRVSVGIEDLEDMKADFTQAFEALLKEDS 299
gene5endoAA     YIFQHAASLGGVESLVEWRAMSSKGEDPLVIRVSVGVEDLEDMKADFTQAFEALLKEDS 299
                **********************************:********************** 
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Figure C.13. Clustal alignment of putative ~300 amino acids encoded by gene 5 on the CD chromosome (Chromosome 14, scaffold 24) and normal chromosome (Nh93153 on Chromosome 4), showing 96% identity at the amino acid level.

Three wks after the soil mix containing pea seedlings was inoculated with 25:75, 50:50, and 75:25 ratios of HT5:HT5-2B07, there was a significant increase ($p < 0.05$) in the relative ratio of HT5-2B07 from the initial ratios of each isolate (Table C.2). However, 3 wks after the pea rhizosphere was inoculated with 25:75, 50:50, and 75:25 ratios of Tr78.2:HT5-2B07, there was no significant change from the initial ratios (Table C.3). Similarly, 3 wks after the pea rhizosphere was inoculated with 25:75, 50:50, and 75:25 ratios of HT5:HT5-2B07Δ3, there was no significant change from the initial ratios (Table C.4). These experiments were repeated with similar results. To determine what might be the reason for the increase in the ratio of HT5-2B07 to HT5, the data were analyzed and observed as a DeWit curve (Fig. C.14).

Transformation of *F. oxysporum* f.sp. *lycopersici, M. grisea*, and *N. crassa* with cosmids 2BO7 and 2F03

All three of these fungi are HUT^- and were transformed with cosmids 2B07 and 2F03. Transformation of *F. oxysporum* f.sp. *lycopersici* isolate 26383, *M. grisea* isolate 70-15, and *N. crassa* isolate 74A with these cosmids yielded ten (2B07 = 8, 2F03 = 2), 24 (2B07 = 3, 2F03 = 21), and five (2B07 = 0, 2F03 = 5) transformants, respectively. None of the transformants obtained for these three fungal species could use HS as a sole C and N source (data not shown).

Table C.2. DNA was extracted from pea rhizospheres 3 weeks post-inoculation with *N. haematococca* MPVI isolate HT5 and transformant HT5-2B07 and the biomass of each isolate was determined using real-time PCR. The actin gene was used to determine total fungal biomass and the *hph* gene to determine the amount HT5-2B07. The means are significantly different from expected results for the 25:75, 50:50, and 75:25 treatments (Newman-Keuls Multiple Comparison Test; $p < 0.05$).

Initial inoculum HT5: HT5-2B07	**3-weeks post-inoculation**
	***hph* as % of *actin* (Mean ± SE)**
0:100	1 ± 1
25:75	36 ± 2
50:50	66 ± 4
75:25	86 ± 3
100:0	100 ± 3

Table C.3. DNA was extracted from pea rhizospheres 3 weeks post-inoculation with *N. haematococca* MPVI transformants Tr78.2 and HT5-2B07 and the biomass of each isolate was determined using real-time PCR. The actin gene was used to determine total fungal biomass and the *PDA1* gene to determine the amount HT5-2B07. The means are not significantly different from expected results for the 25:75, 50:50, and 75:25 treatments (Newman-Keuls Multiple Comparison Test; $p < 0.05$).

Initial inoculum Tr78.2: HT5-2B07	**3-weeks post-inoculation**
	***PDA1* as % of *actin* (Mean ± SE)**
0:100	1 ± 1
25:75	26 ± 3
50:50	52 ± 6
75:25	73 ± 2
100:0	100 ± 4

Table C.4. DNA was extracted from pea rhizospheres 3 weeks post-inoculation with *N. haematococca* MPVI isolate HT5 and transformant HT5-2B07Δ3 and the biomass of each isolate was determined using real-time PCR. The actin gene was used to determine total fungal biomass and the *hph* gene to determine the amount HT5-2B07Δ3. The means are not significantly different from expected results for the 25:75, 50:50, and 75:25 treatments (Newman-Keuls Multiple Comparison Test; $p < 0.05$).

Initial inoculum HT5: HT5-2B07Δ3	**3-weeks post-inoculation**
	***hph* as % of *actin* (Mean ± SE)**
0:100	0 ± 2
25:75	26 ± 4
50:50	48 ± 4
75:25	77 ± 4
100:0	100 ± 5

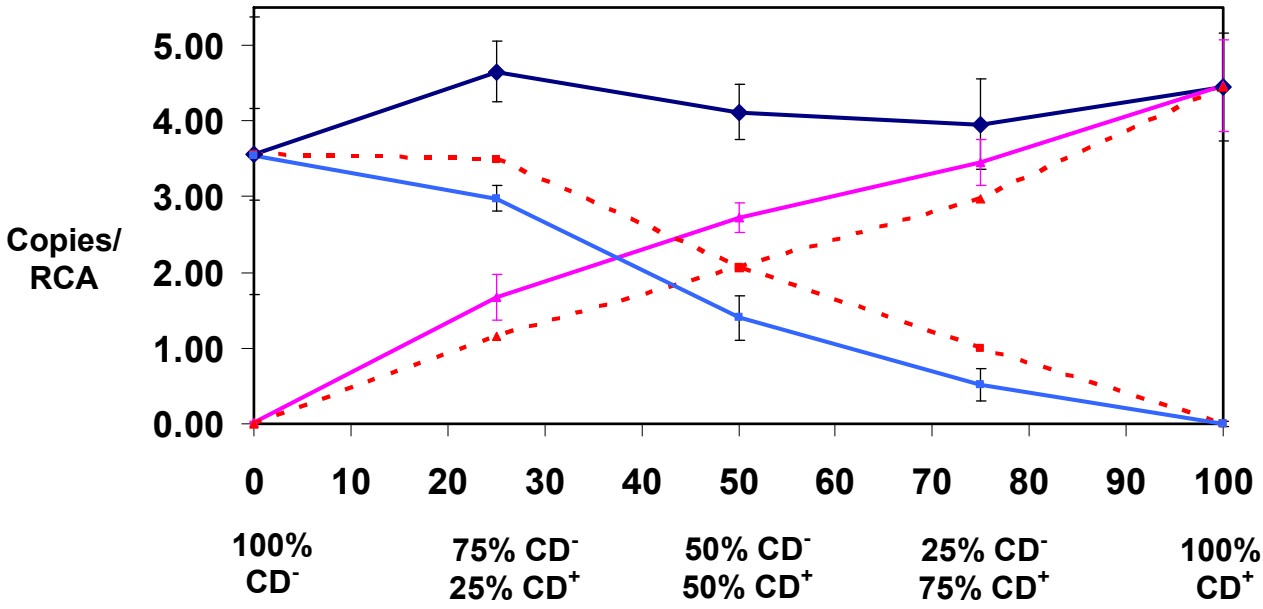

Figure C.14. The deWit Replacement Diagram of data for pea shown in Table C.2. There is better pea rhizosphere colonization by transformant HT5-2B07 (pink line) than by the isolate HT5 (blue line), as indicated by a higher-than-expected ratio of *hph* relative to *actin* gene. HT5-2B07 accounts for 36%, 66%, and 87% of the total fungal biomass 3-weeks post-inoculation with 25:75, 50:50, and 75:25 mixtures. The mean is significantly different from the expected result for the 50:50 treatment (Newman-Keuls Multiple Comparison Test; $p < 0.05$). Three replicates were performed for each treatment.

Discussion

The current studies show that transformation of the CD$^-$ *N. haematococca* isolate HT5 with cosmids 2B07 and 2F03 yields transformants that can utilize HS as a sole C and N source and the subsequent experiments identified a cluster of five genes (hereafter referred to as the *HUT* cluster) on these cosmids that are responsible for the HUT$^+$ phenotype. Previous work (Rodriguez et al. 2008, Appendix A) demonstrated that Tr78.2, which is HUT$^+$, is more competitive in the pea rhizosphere than HT5, which is HUT$^-$. However, Tr78.2 contains ~800 Kb of the *PDA1*-CD chromosome (Rodriguez-Carres 2006) and thus there could be genes, other than the *HUT* genes, that could have accounted for Tr78.2 rhizosphere competency on pea. The results in this current study clearly demonstrate that it is the *HUT* genes that are responsible for the increased rhizosphere competency. The direct comparison of HT5 with HT5-2B07, demonstrate that HT5-B07 was more rhizosphere competent (Table C.2, Figure C.14) and thus this must be due to genes on cosmid B07. Furthermore, Tr78.2 and HT5-B07 were equally competent (Table C.3) and thus the genes on Tr78.2, other than the *HUT* genes, did not increase rhizosphere competency. Finally, there are ~16 additional putative ORFs on cosmid 2B07 in addition to the *HUT* cluster but the observation that HT5 and HT5-2B07Δ3 were equally rhizosphere competent, demonstrates these 16 other genes were not the genes that gave the increased rhizosphere competency of HT5-B07 (Table C.4).

Clusters of functionally-related genes are common for prokaryotic, but not eukaryotic, organisms (Keller and Hohn 1997, Blumenthal 1998, Rosewich and Kistler 2000, Lawrence 2002). Fungal gene clusters have been defined as “the close linkage of

two or more genes that participate in a common metabolic or developmental pathway" (Keller and Hohn 1997). The discovery that genes for certain types of metabolic pathways are clustered in filamentous fungi was first identified by Hull et al. (1989), who found that the four genes for L-proline catabolism in *Aspergillus nidulans* were clustered. Since that time, two major types of "dispensable metabolic pathways", i.e., pathways that are either not required for growth or are only required for growth under a limited range of conditions, have been described: (1) natural product pathways: biosynthetic pathways for low-molecular weight compounds including antibiotics and mycotoxins; and (2) nutrient utilization pathways: catabolic pathways for the utilization of low-molecular-weight nutrients such as proline (Keller and Hohn 1997). Other fungal gene clusters that function in nitrate, sulfur, and iron assimilation acquisition have also been identified (Schierova et al. 2000, Jargeat et al. 2003, Pain et al. 2004, Johnson 2008). Of particular interest to the current study is the *PEP* cluster in *N. haematococca*, a gene cluster that is directly involved with pathogenicity on pea (Han et al. 2001).

Natural product pathways result in the production of structurally diverse secondary metabolites, some of which are involved in specific fungal-plant interactions (Keller and Hohn 1997, Desjardins and Hohn 1997, Walton, 1996). For example, genes involved in the biosynthesis of mycotoxins (e.g., trichothecenes, aflatoxins, paxilline, and sterigmatocystin) and other fungal secondary metabolites (e.g., gibberellins, lovastatin, and β-lactam antibiotics) (Brown 1996, Hohn et al. 1993, Yu et al. 1995, Tudzynski and Holter 1998, Kennedy et al. 1999, Laich et al. 1999, Young et al. 2001, Howlett 2006) are often organized into gene clusters. Nutrient utilization pathways increase the

metabolic versatility of filamentous fungi, thereby enabling them to utilize a variety of compounds such as quinate/shikimate (Geever et al. 1989), proline (Hull et al 1989), *N*-acetylglucosamine (Kumar et al. 2000), and allantoin (Wong and Wolfe 2005) as alternative sources of nutrients. Fungi possess numerous pathways for dispensable metabolic functions and various studies have shown that the genes for these dispensable pathways are often organized in gene clusters (Keller and Hohn 1997). Dispensable metabolic pathways are generally expressed under suboptimal growth conditions and probably function to enhance fungal survival in response to nutrient deprivation or competing organisms (Keller and Hohn 1997). Rosewich and Kistler (2000) suggested that if clustered genes are dispensable, but have adaptive value for colonizing particular ecological niches, then the cluster can be maintained by positive selection in those niches. Natural selection does appear to conserve gene clusters as demonstrated by the cluster of genes for aflatoxin and sterigmatocystin biosynthesis in *Aspergillus* species, where these genes have been maintained as a cluster, despite many internal rearrangements, for at least 120 million years (Cary et al. 2001, Cary and Ehrlich 2006). It has also been suggested that regulatory genes are clustered with structural genes (Keller and Hohn 1997). Although the expression of appropriate catabolic pathways can be vital for survival under conditions of limited nutrients, many of these nutrients are not commonly encountered by fungi. Constitutive gene expression for utilization of these uncommon nutrients would be wasteful of cellular resources. Therefore, fungi have developed regulatory systems to ensure that the required nutrient utilization pathways are expressed in response to changing nutritional conditions, while simultaneously limiting the loss of

cellular resources due to unnecessary constitutive gene expression (Keller and Hohn 1997). Although some clusters contain structural genes as well as those encoding pathway-specific transcription factors and transporters, it is not clear if all gene clusters have all three of these functional gene types (Keller and Hohn 1997).

Interestingly, the putative transcription factor encoded by *HUT2* (as well as the homologous gene on chromosome 10) was found to show high identity with a *PUT3* fungal specific transcription factors. *PUT3* is the specific regulator of the proline-utilization pathway in *Saccharomyces cerevisiae* and is absolutely required for growth on proline (Xu et al. 1995), which, like HS, can serve as either a carbon or a nitrogen source (Brandriss and Magasanik 1979). Like *PUT3* and *GAL4*, a positive regulator of expression of galactose-induced genes of *S. cerevisiae*, *HUT2* has a Zn_2Cys_6 binuclear cluster DNA-binding domain (Todd and Andrianopoulos 1997, Sellick and Reece 2005). It has been previously suggested that transcriptional regulatory genes for some dispensable pathways evolved through the recruitment of functional domains from complementary metabolic pathway genes (Hawkins et al. 1993 and 1994).

Interestingly, three of the five genes identified in the *HUT* cluster (*HUT3, HUT4* and *HUT5*) have homologs on the normal chromosomes in *N. haematococca* MPVI and homologs of these genes are known to be involved in the biosynthesis of methionine, threonine, and isoleucine in plants and microorganisms (Cohen 1983, Mathews and Van Holde 1996, Rodionov et al. 2004). All prototrophic eukaryotic organisms would be expected to have orthologs of these genes and HS serves as an intermediate in the synthesis of all three of these amino acids (Fig. C.15, Mathews and Van Holde 1996).

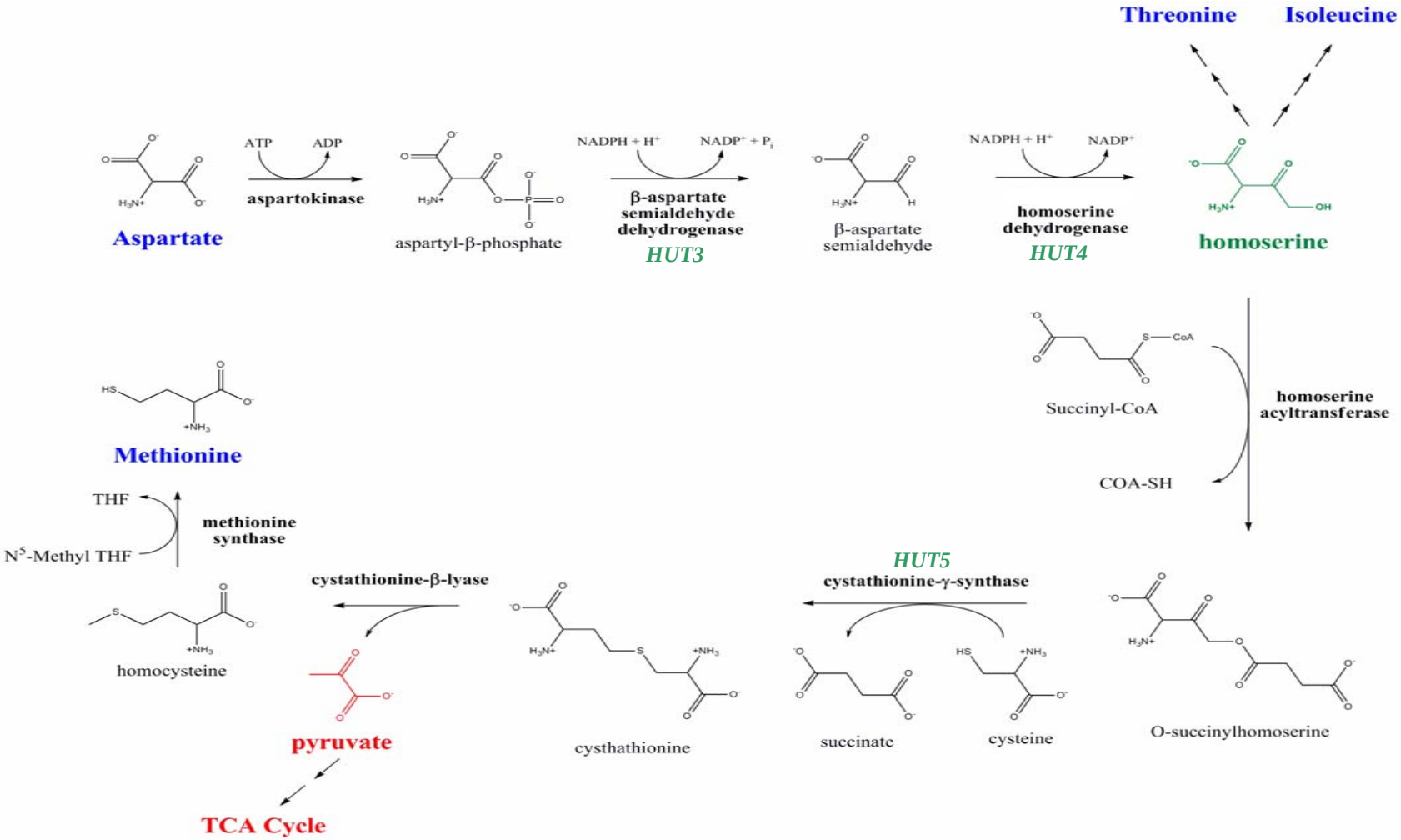

Figure C.15. Biosynthetic pathway for methionine production, showing HS as an intermediary as well as a precursor for the amino acids threonine and isoleucine. During methionine biosynthesis, pyruvate, which can be directed to the TCA cycle, is also produced. Homologs to *HUT3*, *4*, and *5* are indicated in green.

The first reaction in the synthesis of methionine (Fig. C.15) is the phosphorylation of aspartate to aspartyl-β-phosphate via aspartokinase. β-aspartate-semialdehyde dehydrogenase (a homolog of *HUT3* and gene Nh79751 on chromosome 4) dephosphorylates the aspartyl-β-phosphate to β-aspartate-semialdehyde, which is then reduced to HS by homoserine dehydrogenase (a homolog of *HUT4* and gene Nh93154 on chromosome 4). Homoserine acyltransferase catalyzes the reaction between HS and succinyl-CoA to form O-succinylhomoserine, which then reacts with cysteine to produce cystathionine via cystathionine-γ-synthase (a homolog of *HUT5* and gene Nh93153 on chromosome 4). Cystathionine is cleaved by cystathionine-β-lyase to yield homocysteine, which is methylated by methionine synthase to methionine. Although the gene for HS acyltransferase, the enzyme that converts HS to O-succinylhomoserine, is not clustered with the other five *HUT* genes on the CD chromosome, there are at least four other putative copies of this gene on *N. haematococca* MPVI chromosomes 5, 7, and 8. Although the ORFs for *HUT3*, *4*, and *5* are very similar to those on the normal chromosomes, the regions upstream from the ORFs containing the promoter regions differ (Fig. C.8), indicating that they may be regulated differently. Unfortunately, these three *HUT* genes are so similar to the putative biosynthetic genes found on the normal chromosomes that it was not possible to measure the individual expression of the different genes. It is interesting to note that, in the current study, genes encoding a putative transposase and a putative reverse transcriptase are located just downstream from *HUT5* (data not shown) suggesting a possible means of duplication and movement of *HUT* genes within the genome of *N. haematococca*.

It is not clear how the *HUT* genes allow *N. haematococca* MPVI to utilize HS as a sole C and N source. Clearly, homologs for all but one of the *HUT* genes are present in this fungus and those on the normal chromosomes are presumed to be involved in the synthesis of threonine, isoleucine and methionine. The extra copies of these biosynthetic genes might allow extra production of the amino acids from HS beyond what is normally needed for protein biosynthesis. The reaction whereby cystathionine-β-lyase converts cystathionine to homocysteine yields pyruvate, which can be converted to acetyl-CoA and fed into the TCA cycle and may be means to obtain additional energy and carbon molecules for growth. However, determination of how the genes on the *HUT* cluster function biochemically needs further research.

Although the *HUT* cluster genes did restore the ability to use HS when transformed into an isolate of *N. haematococca* MPVI lacking the cluster, it did not give that ability to three other fungi that lack the HUT trait. Thus, it would appear that there are additional genes in *N. haematococca* needed for demonstrating the HUT trait and that they are lacking in these other fungi or the *HUT* genes were not expressed in the other fungi.

Previous studies have shown that the *PEP* cluster of *N. haematococca* MPVI isolate 77-13-4 have properties that are consistent with an origin by horizontal gene transfer: (1) unusual codon usage and GC content compared to genes found on the normal chromosomes and this difference is similar to genes found on bacterial “pathogenicity islands” and “symbiosis islands”, clusters of genes that have been acquired by horizontal gene transfer (Hacker et al. 1997, Hacker & Carniel 2001, Ochman et al. 2000); (2)

dispensability (Temporini and VanEtten 2002); (3) nonhomogeneous distribution within members of the same species (Temporini and VanEtten 2002); and (4) discontinuous phylogenetic distribution (Temporini and VanEtten 2004). Subsequent comparisons of the *N. haematococca* genome to *Fusarium graminearum (Gibberella zeae),* the most closely related sequenced fungus, has identified 6,785 genes unique to *N. haematococca* (Coleman 2008). A comparison of the unique genes to the orthologs have shown a codon bias for certain amino acids (Coleman 2008) and a comparison of codon usage between *HUT4*, and the orthologous homoserine dehydrogenase has demonstrated it also has this codon bias, which would be consistent with HGT (Jeff Coleman, pers. comm.). It was not possible to make this comparison to the homologs of the other *HUT* genes as the normal ortholog could not be established to make the comparison. The rhizosphere and the soil environments are habitats that are conducive to horizontal gene transfer between bacteria (Droge et al. 1999, Espinosa-Urgel 2002, Lynch et al. 2004, van Elsas & Bailey 2002). Similarly, it has been proposed that fungal pathway gene clustering results from the acquisition of these pathways from prokaryotes, whose metabolic pathways are generally clustered, via horizontal gene transfers (Keller and Hohn 1997, Rosewich and Kistler 2000). It has also been suggested that the clustering of genes facilitates horizontal gene transfer of genes involved in the same metabolic pathway (Lawrence 1999, Walton 2000). One concrete example of gene cluster transfer between fungi is that of the *ACE1* gene cluster of *Aspergillus clavatus*, which appears to have originated by horizontal transfer from a donor closely related to *M. grisea* (Khaldi et al. 2008), indicating that the horizontal transfer of gene clusters is indeed possible in fungi. Although the genes in the

HUT cluster lack features typically found in prokaryotes (i.e., arrangement into operons and lacking introns), some of the features are consistent with the cluster arising by HGT from another eukaryotic organism. Rhizosphere competition assays showed that the *HUT* cluster gives *N. haematococca* a competitive advantage in the rhizosphere of pea plants (Table C.2, Figure B.14). As mentioned previously, the *PDA1* CD chromosome of *N. haematococca* MPVI can be compared to *sym* plasmids of rhizobacteria. For example, the *sym* plasmid of *Rhizobium leguminosarum* has *NOD* genes for colonization of host-plant roots (Rosenberg et al. 1981, Martinez-Romero and Caballero-Mellado 1996), as well as genes for catabolizing unique compounds present in host-plant root exudates (Rosenberg et al. 1981, Boivin et al. 1991, Goldmann et al. 1994). Direct correlations exists between the ability of *R. leguminosarum* biovar *viciae* to nodulate pea, and its ability to catabolize HS (Economou et al. 1988) and between the ability of *Sinorhizobium meliloti* to nodulate alfalfa, and its ability to catabolize stachydrine, a compound present in alfalfa root exudates (Goldman et al. 1991, Phillips et al. 1995). It has been demonstrated for *S. meliloti* that the ability to use stachydrine gives this bacterium a competitive advantage in the rhizosphere of alfalfa (Phillips et al 1996). Therefore, since the *PDA1* CD chromosome of *N. haematococca* MPVI carries genes (the *PEP* cluster) required for pathogenicity on pea (Han et al. 2001, Wasmann and VanEtten, 1996) as well as genes (the *HUT* cluster) that give it a competitive advantage in pea rhizosphere (Appendix A, Rodriguez et al. 2008), it is reasonable to conclude that the *PDA1* CD chromosome is functionally analogous to the *sym* plasmids of rhizobacteria.

It is not known exactly where the *N. haematococca* MPVI is growing in the rhizosphere in the present study, but previous research has shown that this fungus grows as a mantle on the border cells that surround the root tips of pea (Gunawardena and Hawes 2002). It has also been demonstrated that HS is present in pea root tips and that substantial amounts are released from the site of secondary root emergence (Van Egeraat 1975a, 1975b). Since the change in relative biomass of HT5 to HT5-2B07, as shown in the DeWit curve (Fig. C.14), was due to a decrease in HT5 biomass and an increase in HT5-B07 biomass from what was expected, it implies that there was competition between the two isolates. The observed competition could be explained by growth at the same site where HS was present along with other nutrients. Studies that examine where the HUT^- and HUT^+ isolates are growing should resolve this question. Nevertheless, the current study establishes that HUT can be a rhizosphere competency trait for a fungus and appears to be the first example of a rhizosphere competency trait being identified in a fungus.

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