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Identification and expression analysis of sugar transporters in microbotryum violaceum.

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Identification and Expression Analysis of Sugar Transporters in *Microbotryum violaceum*

By

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Introduction

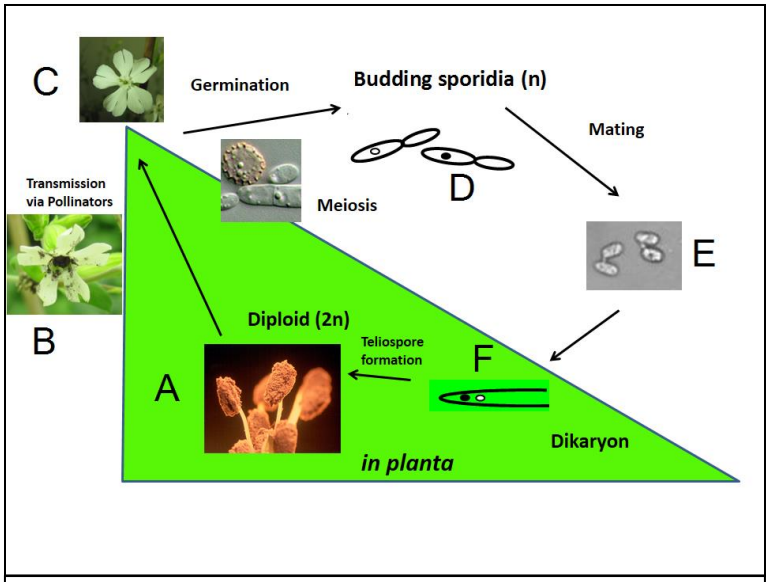


Fig 1. The life cycle of the anther smut fungus. *M. violaceum* diploid teliospores are produced in the anthers (a) and are transmitted by pollinators (b) onto a healthy plant (c). The teliospores germinate, undergo meiosis, and produce yeast-like sporidia (d). Conjugation takes place on the plant between sporidia of opposite mating types (e). Dikaryotic hyphae grow in the plant (f) and overwinter in vegetative tissues. The following year, infection is systemic, and all flowers produce teliospores (a).

Microbotryum violaceum is a heterobasidiomycete fungal pathogen that infects *Silene latifolia*, a dioecious wildflower species. *M. violaceum* is an obligate biotroph, meaning that it requires the host plant living tissue in order to grow; as such, it must infect a host to complete its life cycle and must do so without killing the host. Infection sterilizes the plant, and in male plants, replaces the pollen grains with teliospores (Fig 1). In female plants, the fungus invokes the growth of stamen and anthers on which to place its teliospores. In either male or female (pseudomale) flowers, the teliospores may then be spread to uninfected plants by insect pollinators [1]. Haploid cells of *M. violaceum* contain one of two mating types, A1 or A2, and cells of opposite mating type must mate to produce the form of the fungus necessary for infection to occur.

Most model systems for fungal host/pathogen interactions are within agricultural systems [2]. *M. violaceum* provides an opportunity to study host/pathogen interactions in a "wild", less manipulated setting, where host populations may not be as genetically similar as those for agricultural pathogens [1, 2]. *M. violaceum* can also serve as a model for the study of host shifts by infectious diseases [2]. This system also allows for further study of genes that may be necessary for pathogenicity [8]. Though a number of pathogenicity genes have already been discovered for phytopathogenic fungi [9, 10], those required for pathogenicity of *M. violaceum* are still unknown.

Background

A draft genome sequence at 18x coverage was produced for a haploid strain derived from meiosis of teliospores isolated from the host *Silene latifolia*. The draft sequence is currently in the process of annotation and is publicly available through a website from the Broad Institute of MIT and Harvard (http://www.broadinstitute.org/annotation/genome/Microbotryum_violaceum/MultiHome.html). Using Illumina Next Gen sequencing, deep transcriptome information is being generated about a variety of stages in the lifecycle of the fungus, with particular emphasis on the late stages of infection, when teliosporogenesis occurs. This means that "global" gene expression information is being generated by this method for essentially all predicted *M. violaceum* genes during each of the stages being examined.

As presented in recent publications, sugar transporters in fungi have been shown to affect fungal virulence in plants, particularly in the case of biotrophic fungi such as *Ustilago maydis* and several species of rust fungi [11, 12, 17, 18]. Specifically, the *srt1* gene in *U. maydis* was found to code for a high-affinity, sucrose-specific transporter that likely provides advantages for the acquisition of carbon after the infection of host tissue as well as helping to prevent plant defense mechanisms from being induced. Bringing sucrose into the fungal cell rather than breaking sucrose down outside of the cell and transporting the resulting simple sugars into the cell is less likely to be recognized by the plant [18]. The *hxt1* gene in the rust fungus *Uromyces fabae* codes for a hexose transporter that may fulfill a similar role, as it is upregulated in rust haustoria, an important fungal organ during infection [17].

The importance of sugar transporters for pathogenicity of *M. violaceum* is unknown. Thus, identifying and determining possible roles in pathogenicity of these sugar transporters is of interest. Such work may give insight both as to how the fungus initially infects its host and how this infection is maintained in order for the fungus to complete its life cycle [9].

Methods

Initial Bioinformatic Analyses and Target Gene Selection

Potential sugar transporter genes of *Microbotryum violaceum* were identified by comparing the *srt1* and *hxt1* genes from *U. maydis* and *U. fabae,* respectively, with the *M. violaceum* genome and proteome through the use of BLASTP and BLASTN (Protein/Nucleotide Basic Local Alignment Search Tool). BLAST is a readily accessible online bioinformatics tool that compares DNA, RNA, or amino acid sequences to databases of similar sequences, such as genomes, transcriptomes, and proteomes. Genes that have some degree of similarity to the entered sequences are listed as results. To assure that potential hits from BLAST are likely sugar transporters, other data were also examined, including predicted subcellular localization of the protein and conserved functional domains within each predicted protein. TargetP1.1, SignalP3.0, SignalP4.0, TMHMM2.0, PredGPI, Phobius, Prosite and WoLF PSORT were used to ensure that the predicted sugar transporters are located within the cell membrane. UniProtKB and FunSecKB scores of the closest BLAST hit were used for confirmation when there were contradictions in the prediction and then subcellular localizations were manually curated. Positive indicators of a predicted sugar transporter include conservation of the sugar transporter domain and high numbers of predicted transmembrane helices, which strongly suggest that the protein is located in the plasma membrane.

Once the sugar transporters were identified, several of them were selected for closer study based on Orthofam ID uniqueness, information yielded from National Center for Biotechnology Information (NCBI) BLAST hits against other genomes, and initial expression data already collected through RNASeq, which provides RNA quantity from a genome at a certain point in time, such as a certain stage of the life cycle [6]. The initial expression data (Table 2) were taken from the fungus isolated from three different growth conditions – haploid cells on rich agar, haploid cells on water agar, and late stage infection (i.e., dikaryotic or diploid fungus) in male floral tissue of *S.*

latifolia.

RNA Purification and Manipulation

Liquid nitrogen grinding was used to prepare the cells for RNA isolation, which was done using the RNeasy™ Plus Mini Kit (Qiagen, Venlo). Illumina sequencing was used to analyze gene expression levels in three RNA sequencing experiments, as follows:

Rich: 4-day old yeast-like haploid cells, p1A1 and p1A2 (Fig 1d), grown separately on rich media.

Water: 4-day old yeast-like haploid cells, p1A1 and p1A2 (Fig 1d), grown separately on rich media, then placed separately on nutrient-free media at 14˚C for 3 days.

MI late: 4 mm to fully bloomed, male infected host floral tissue (Fig 1a), containing diploid fungus undergoing teliosporogenesis.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) using Invitrogen™ SuperScript III First-Strand Synthesis System (Life Technologies) was then performed on the RNA samples extracted from *M. violaceum* grown in the three conditions used for the initial analysis, as well as when mated on water agar. This last condition was another unique aspect of the study, as mating is a prerequisite for pathogenic development; when exposed to a suitable host plant, the mated cells further differentiate into a dikaryotic filamentous form that penetrates the plant [1]. The isolated RNA samples were then DNase-treated (Ambion Turbo DNA-*free*™ Kit) to remove all traces of DNA from the sample. The reverse transcriptase reaction created cDNA from the RNA. This cDNA product from the reverse transcriptase reaction was then tested for quality and full degradation of genomic DNA (gDNA) using standard PCR and agarose gel electrophoresis. Polymerase Chain Reaction is a reaction that repeatedly and selectively replicates a segment of DNA that is flanked by small DNA primers to allow for replication. The samples were then subjected to an agarose gel by the application of field electrophoresis to verify their size and the absence of contamination by other DNA.

Expression Analysis Using Quantitiative Real-Time Polymerase Chain Reaction (qPCR)

Quantitative real-time PCR (qPCR) was then performed on each gene for every condition. qPCR is the same reaction as standard PCR, but a dye is added that fluoresces when bound to double-stranded DNA. As more and more double-stranded DNA is made during PCR, this increasing fluorescence is measured and the instrument readings allow the relative expression of various genes to be determined [16]. These experiments utilized SYBR Green dye and the Applied Biosystems StepOne Real-Time PCR system.

MVLG_00180 (beta tubulin) was used as the endogenous control, because it is consistently expressed under all of the growth conditions. The optimal primer (62.5 nM) was determined, and triplicate samples and water negative controls were used for each gene. The cDNA obtained from the haploid strain, p1A1, grown on rich media was used as the reference to which all of the other conditions were compared [4]. 1.6 µl of each 2.5 µM primer, 6.4 µl of cDNA template, 32 µl of Power SYBR Green PCR Master Mix (Applied Biosystems), and 32 µl of water was used for each triplicate to yield 20 µl reactions. The thermal cycling comprised an initial step at 95˚C for 10 minutes and 40 cycles of 95˚C for 15 seconds and 60˚C for 1 minute. The difference in the fluorescence signal between MVLG_00180 and each gene of interest was measured with cDNA prepared from the fungus under each growth condition. This fluorescence difference between each gene and MVLG_00180 was then compared to the same difference in the reference sample (p1A1 rich cDNA). These comparisons show whether the gene is differentially expressed under each growth condition relative to the p1A1 type grown on rich agar [14]. This is known as the comparative CT method of analyzing qPCR data. The equations used to generate the log fold change were (-LOG(1/(2^(-N))) for positive values and (LOG(2^(-F6))) for negative values. These relative expression data serve as validation of the initial expression data collected through RNASeq and adds data for the mated fungus.

Transformation of M. violaceum and Over-Expression of Target Genes

Differential expression of genes between the conditions has prompted ongoing transformation experiments in order to overexpress genes in certain conditions to view morphological and developmental changes. These observations may give insight as to the role of the gene in pathogenicity and/or mating [5].

Overlap PCR was performed for each selected gene to create a construct with the appropriate promoter for the condition, such as overexpression of the gene in the fungus grown on water agar. After viewing the RNASeq data for the respective conditions, candidate promoters were chosen from *M. violaceum* genes that were found to be among the most highly expressed on water agar or on rich media. For amplification of each sugar transporter gene coding sequence fragment, the cycling conditions comprised an initial step at 94˚C for 4 minutes, followed by 35 cycles of denaturing at 94˚C for 30 seconds, annealing at 60˚C for 30 seconds, and extension at 72˚C for 2 minutes and 10 seconds, followed by a terminal extension at 72˚C for 5 minutes. The cycling conditions for amplification of the promoter fragment were the same as for the gene coding fragment, but with a 1 minute and 10 second extension rather than a 2 minute and 10 second extension each cycle. For both of these PCR experiments, 1.25 µl of the forward and reverse primers, 2.5 µl 10x Ex Taq buffer, 2.0 µl dNTP mixture, 16.875 µl H20, 0.125 µl Ex Taq Polymerase (Takara Bio), and 1.0 µl of the DNA template (cDNA for coding sequences of genes, gDNA for promoter sequences) were used for each 25 µl reaction.

For the overlap PCR reaction, 2.0 µl of the forward and reverse primers (table 1), 5.0 µl 10x Ex Taq buffer, 4.0 µl dNTP mixture, 31.25 µl H20, 0.25 µl Ex Taq Polymerase (Takara Bio), 0.5 µl of the promoter fragment DNA template, and 5.0 µl of the gene coding fragment were used for each 50 µl reaction. Cycling conditions comprised an initial step at 94˚C for 4 minutes, followed by 35 cycles of denaturing at 94˚C for 30 seconds, annealing at 60˚C for 30 seconds, and extension at 72˚C for 3 minutes and 30 seconds, followed by a terminal extension at 72˚C for 5 minutes. All fragments were recovered from agarose gels by using the Zymoclean™ Gel DNA Recovery Kit.

These constructs will be inserted into a TOPO TA pCR2.1 vector (Life Technologies) that is optimized for cloning PCR products in *E. coli*. After identifying clones, the constructs will be subsequently cut out and introduced into similarly digested vector for transformation of *M. violaceum*. The transformed cells will then be grown under the appropriate conditions and observed. Ideally, some phenotypic change will occur to allow conclusions concerning the function of the gene in development/pathogenicity to be made.

Table 1. Primer sequences used to create coding sequence and promoter fragments for overlap PCR. Primers were designed using Primer3 software.

Target	Primer Sequence
MV05589F	AATTAATTAA|GCTGTATAGGGGGTTCGTGA
MV07006F$_{RC}$_MV05589R	TTGTGTAATGATGGCTTGGC|AGAGAGGATGCGATCGAGTG
MV05589R$_{RC}$_MV07006F	CACTCGATCGCATCCTCTCT|GCCAAGCCATCATTACACAA
MV07006R	AAAAGGACGGAGCGAGAAAG
MVLG06949F	AATTAATTAA|CCACCCACAGAACAACTCAA
MVLG07006FRC _MVLG06949R	TTGTGTAATGATGGCTTGGC|TCTTCAGGGTCGTTCCCTCT
MVLG06949RRC_MVLG07006F	AGAGGGAACGACCCTGAAGA|GCCAAGCCATCATTACACAA
MVLG00507FRC_MVLG06949R	GCAAGACATGGCCTCTCA|TCTTCAGGGTCGTTCCCTCT
MVLG06949RRC_MVLG00507F	AGAGGGAACGACCCTGAAGA|TGAGAGGCCATGTCTTGC
MVLG00507R	GAACCACACGCAGACCTAAA

Results and Discussion

Initial Bioinformatic Analyses and Target Gene Selection

Of the 64 *M. violaceum* genes that contain the sugar_tr domain (PF0083), 25 were also BLASTP hits to Srt1 (Genbank: XP_758521) of *Ustilago maydis* and Hxt1 (Genbank: CAC41332) of *Uromyces fabae*. Of these 25 predicted sugar transporters, four were up-regulated in MI late. Two were down-regulated in MI late. Two were up-regulated and two down-regulated in Water. One was up-regulated and one down-regulated in Rich. One was upregulated in Water relative to MI late. One was upregulated in Rich relative to MI late, and one was upregulated in Water relative to Rich. Nine were not differentially expressed. Of these 25 that were hits to Srt1 or Hxt1, 7 were selected for additional study based on OrthfamID uniqueness, differential expression in initial

analysis, and information yielded from BLAST hits to the NCBI database (Table 2, Table 3).

Table 2. Rationale for choosing each gene described in detail.

Gene Name	Rationale for Choosing
MVLG_07006	Upregulated in MI late. NCBI protein BLAST gives reason to believe it is a maltose permease. Part of maltose permease family.
MVLG_05093	Upregulated in MI late. NCBI protein BLAST gives reason to believe it is a lactose permease.
MVLG_04801	Unique orthofam ID, localization uncertain. NCBI protein BLAST yield no further information other than that it is a sugar transporter.
MVLG_06292	Unique orthofam ID. NCBI protein BLAST gives reason to believe it is an iron permease and/or maybe a multidrug transporter, though it belongs to the sugar transporter family as well. Gene is also in the fungal trichothecene (myotoxins produced by some fungi, such as some species of *Fusarium*) efflux pump family.
MVLG_06941	Downregulated in MI late. NCBI protein BLAST gives reason to believe it is a monosaccharide transporter, no indication of specificity.
MVLG_00507	Downregulated in MI late. NCBI protein BLAST gives reason to believe it is a phosphate transporter or permease. Part of phosphate H+ symport family as well as sugar transporter family.
MVLG_05629	Upregulated in water. NCBI protein BLAST yielded no further information other than that it is a sugar transporter.

Table 3. Descriptive matrix from RNASeq data showing initial expression data of each gene.

Gene Name	water.rsem	rich.rsem	milate.rsem	w_v_r logFoldChange	w_v_mi logFoldChange	r_v_mi logFoldChange	Regulation
MVLG_07006	8	10	1043	ns	-8.583	-8.4558	Up in MI Late
MVLG_05093	1	5	615	ns	-10.8209	-8.6937	Up in MI Late
MVLG_04801	42	19	19	ns	ns	ns	ns
MVLG_06292	125	56	39	ns	ns	ns	ns
MVLG_06941	1605	2075	4	ns	7.09186	7.2676	Down in MI Late
MVLG_00507	3841	13249	91	ns	3.8429	5.4345	Down in MI Late
MVLG_05629	1264	166	44	3.0851	3.2878	ns	Up in Water

Rsem data shows transcript copy number for each gene in each condition. The logFoldChange shows the change in expression of the gene, if any, between the two conditions. For instance, the w_v_r logFoldChange column compares expression in water to that in rich. The Regulation column describes the differential expression of the gene, if any, based on the greatest magnitude logFoldChange. "ns" stands for "Not Significant".

Expression Analysis Using Quantitiative Real-Time Polymerase Chain Reaction (qPCR)

The inventory of predicted proteins, differentially expressed, provides insights into critical aspects of the unique biotrophic lifestyle of *M. violaceum*. *M. violaceum*, despite being a biotrophic pathogen, actually causes necrosis in floral tissue, preventing the flower from creating pollen in male plants and suppressing growth of the ovary and styles in female plants [10]. Some logical predictions and conclusions can be made about sugar transporters that may play a role in this process from the relative expression data gathered (Table 4, Figs 2.1-2.5) [5, 17, 18].

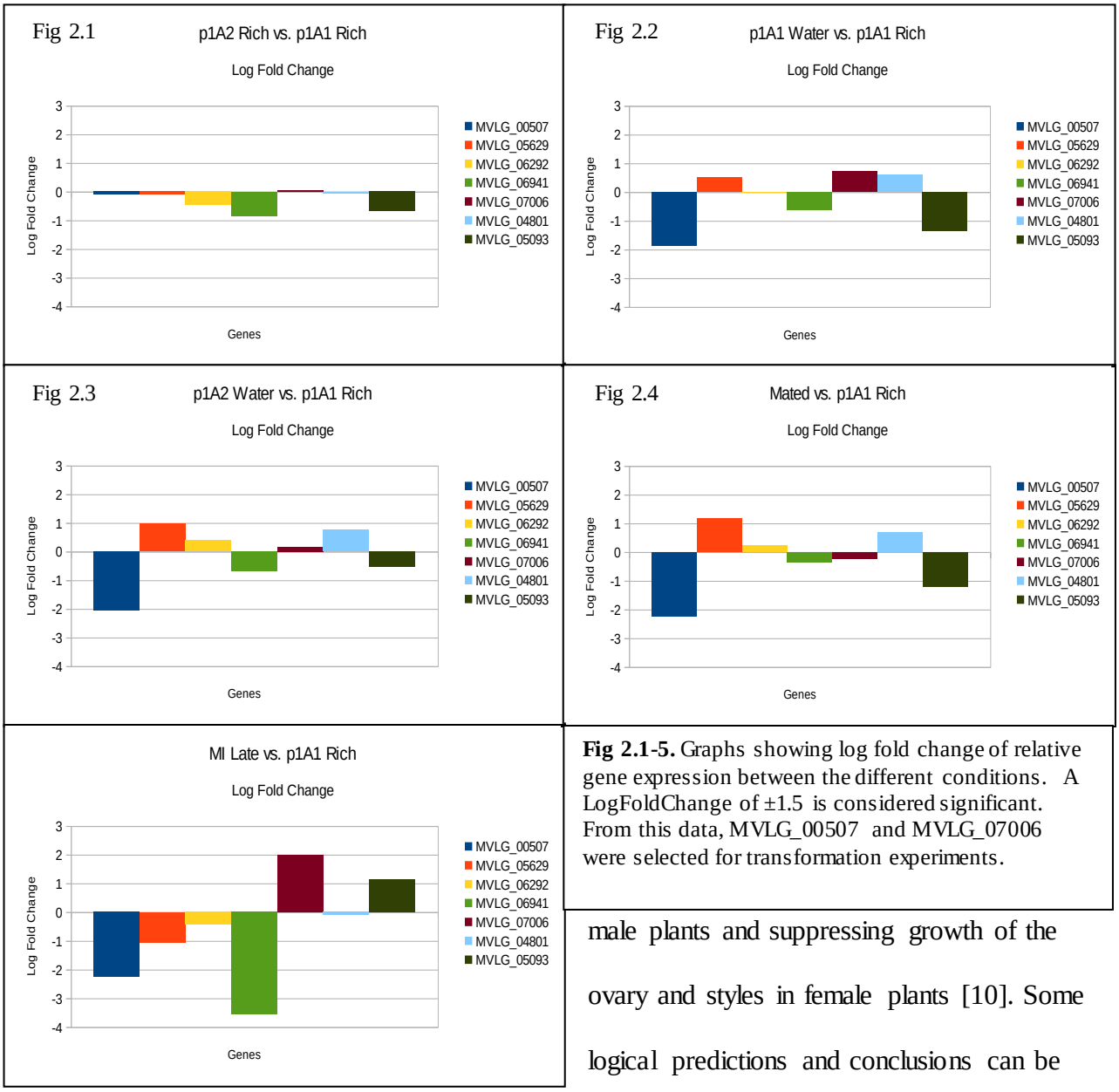


Fig 2.1-5. Graphs showing log fold change of relative gene expression between the different conditions. A LogFoldChange of ±1.5 is considered significant. From this data, MVLG_00507 and MVLG_07006 were selected for transformation experiments.

The predicted proteins that are down-regulated in MI late (MVLG_00507, MVLG_06941) may hinder the progression of the fungus into the next stage of its life cycle (teliosporogenesis), may induce a defense mechanism from the host or both [13, 15, 18]. The predicted proteins that are

up-regulated in MI late may be involved in host invasion and evasion of physical and chemical defense system [9]. It would also make sense that it may scavenge nutrients from the tissue damaged by the fungus [7, 10, 19]. The predicted proteins that are up-regulated in Mated (MVLG_5629) may be necessary for the fungus to mate properly and progress to the next stage of its life-cycle (dikaryon formation). The predicted proteins that are down-regulated in Water (MVLG_05093) may cause problems with cell-cell communication, hindering the mating process [20]. The predicted proteins that are up-regulated in Water (MVLG_06292, MVLG_04801) may possibly be involved in preparation for mating and associated responses to nutrient starvation [3].

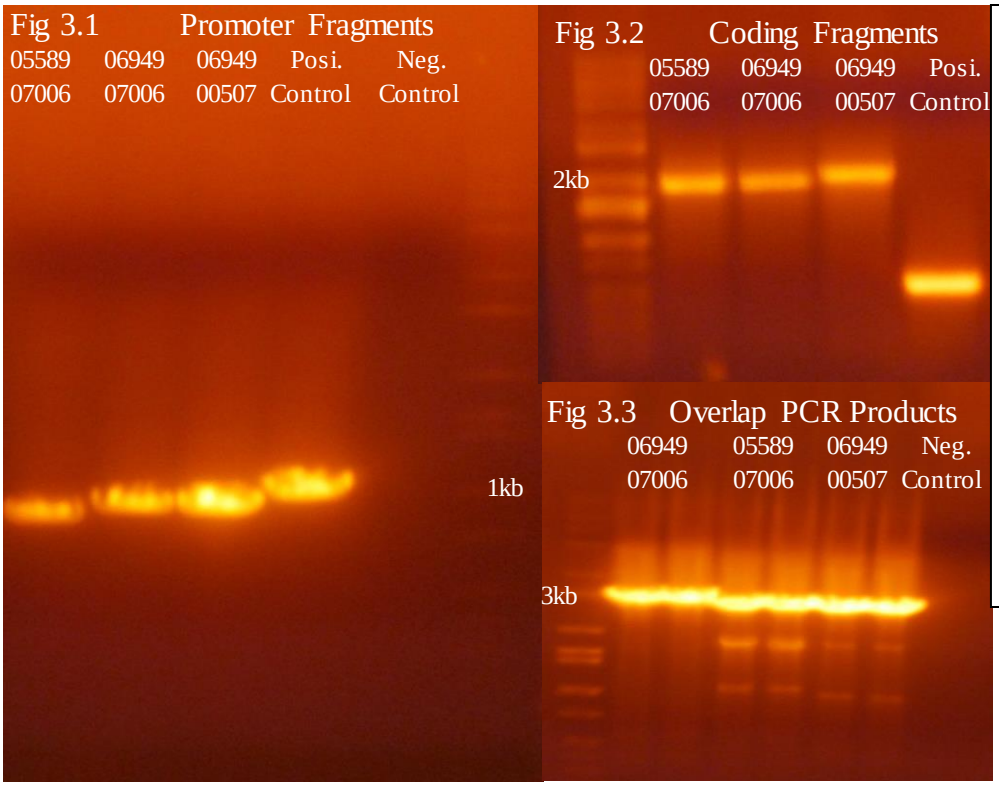
Table 4. Descriptive matrix showing the log fold change data presented in Figures 2.1-4. The Regulation column describes the differential expression of the gene based on the greatest magnitude LogFoldChange.

Gene	LogFoldChange p1A2r_v_p1A1r	LogFoldChange p1A1w_v_p1A1r	LogFoldChange p1A2w_v_p1A1r	LogFoldChange mated_v_p1A1r	LogFoldChange MILate_v_p1A1r	Regulation
MVLG_00507	-0.1048779419	-1.8645737725	-2.0541465092	-2.2325185151	-2.2480929107	Down in MI Late
MVLG_05629	-0.1076872342	0.4925091553	0.9629311378	1.1732680961	-1.0521148863	Up in Mated
MVLG_06292	-0.4581478596	-0.0008483025	0.3954950145	0.2348517991	-0.4262286719	Down in p1A2 Rich
MVLG_06941	-0.8460289926	-0.6320859272	-0.6779951088	-0.3585392283	-3.5640744356	Down in MI Late
MVLG_07006	0.0551486656	0.7170450208	0.1657039479	-0.2314229444	1.974981942	Up in MI Late
MVLG_04801	-0.0583712978	0.585467218	0.7577496948	0.685778389	-0.101010314	Up in p1A2 Water
MVLG_05093	-0.6721780051	-1.3426365269	-0.5188236924	-1.2076766817	1.1524511942	Down in p1A1 Water

Transformation of M. violaceum and Over-Expression of Target Genes

Constructs have been made from promoters of genes that are very highly expressed in the fungus grown in rich (MVLG_05598) or water (MVLG_06949) media and two of the genes that showed the greatest differential expression, MVLG_00507 and MVLG_07006 (Table 4, Figs 2.1-2.5, 3.1-3.3). As MVLG_07006 is upregulated in MI Late, the effects of upregulating it in water or rich media may show why it is not as highly expressed in earlier stages of development or during mating. Similarly, MVLG_00507 is heavily down regulated in MI Late, mated, and water; upregulating it in those conditions may provide insight as to why it is so upregulated in rich media.

Attempts to clone the constructs into TOPO vectors and insert them into *E.coli* are ongoing. Once this is achieved, the constructs will be subcloned into an appropriate vector and used for the transformation of *M. violaceum*. Potential transformants will be confirmed for overexpression of the appropriate gene by qPCR. The transformants will then be assessed for any morphological or developmental changes when grown in the appropriate condition.



Figures 3.1-3.3. Agarose gel images showing the creation of the overlap PCR products (3.3). Figure 3.1 shows amplification of the promoter fragments. Figure 3.2 shows the amplification of the coding fragments. Figure 3.3 shows the resulting constructs when the two fragments are combined using overlap PCR.

There are a number of predictions that can be made about the experimental outcomes. If a gene is typically downregulated in mated cells (such as MVLG_00507), it seems reasonable that overexpressing the gene in the fungus grown on water agar (a condition that normally promotes mating) may interfere with mating either by a lower frequency of mating or morphological change in cells that hinders mating. Similarly, if a gene that is normally downregulated in fungus grown in rich media is overexpressed, filamentation may be induced or growth accelerated. Conversely, if a gene is normally upregulated in rich media in relation to all other conditions (such as MVLG_07006), it can be hypothesized that the sugar transporter may be specific for the sugar present in the media (glucose in our experiments) or may inhibit mating in some other way or both. Overexpression of this gene on water agar may lead to an inhibition of mating.

Future Work

The transformation experiments will direct the next logical to examine the many aspects of sugar transporters in *M. violaceum*. *M. violaceum* transformants, especially those with phenotypic changes, will again be characterized for levels of expression or their respective "trans"-gene to make certain that there has indeed been an increase in expression relative to the wild type strains. Additional experiments to look at expression levels during other stages of development within the plant (e.g., directly after infection, earlier stages of bud development, etc.) will provide insight into the role sugar transporters play in earlier stages of infection or evasion of plant defenses. Expression experiments with the fungus grown on media containing different types of sugars may also yield information about the specificity of each sugar transporter and the regulation of their genes, as well as suggesting additional specific roles to be tested in the developmental stages of this fungus.

References

1. Alexander HM: **An Experimental Field Study of Anther-Smut Disease of Silene alba Caused by Ustilago violacea: Genotypic Variation and Disease Incidence**. *Evolution* 1989, **43**(4):835-847.
2. Antonovics J, Hood ME, Partain J: **The Ecology and Genetics of a Host Shift: Microbotryum as a Model System**. *The American Naturalist* 2002, **160**(S4):S40-S53.
3. Bahn Y-S, Xue C, Idnurm A, Rutherford JC, Heitman J, Cardenas ME: **Sensing the environment: lessons from fungi**. *Nature Reviews Microbiology* 2007, **5**(1):57-69.
4. Bustin Sxaa, Mueller R: **Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis**. *Clinical Science* 2005, **109**:365-379.
5. Chen L-Q, Hou B-H, Lalonde S, Takanaga H, Hartung ML, Qu X-Q, Guo W-J, Kim J-G, Underwood W, Chaudhuri B *et al*: **Sugar transporters for intercellular exchange and nutrition of pathogens**. *Nature* 2010, **468**(7323):527-532.
6. Chu Y, Corey DR: **RNA sequencing: platform selection, experimental design, and data interpretation**. *Nucleic acid therapeutics* 2012, **22**(4):271-274.
7. Divon HH, Fluhr R: **Nutrition acquisition strategies during fungal infection of plants**. *FEMS microbiology letters* 2007, **266**(1):65-74.
8. Hughes CF, Perlin MH: **Differential expression of mepA, mepC and smtE during growth and development of Microbotryum violaceum**. *Mycologia* 2005, **97**(3):605-611.
9. Idnurm A, Howlett BJ: **Pathogenicity genes of phytopathogenic fungi**. *Molecular Plant Pathology* 2001, **2**(4):241-255.
10. Mendgen K, Hahn M: **Plant infection and the establishment of fungal biotrophy**. *Trends in Plant Science* 2002, **7**(8):352-356.
11. Ozcan S, Johnston M: **Three different regulatory mechanisms enable yeast hexose transporter (HXT) genes to be induced by different levels of glucose**. *Molecular and Cellular Biology* 1995, **15**(3):1564-1572.
12. Özcan S, Johnston M: **Function and Regulation of Yeast Hexose Transporters**. *Microbiology and Molecular Biology Reviews* 1999, **63**(3):554-569.
13. Rolland F, Baena-Gonzalez E, Sheen J: **Sugar sensing and signaling in plants: conserved and novel mechanisms**. *Annu Rev Plant Biol* 2006, **57**:675-709.
14. Schmittgen TD, Livak KJ: **Analyzing real-time PCR data by the comparative CT method**. *Nature protocols* 2008, **3**(6):1101-1108.
15. Sheen J, Zhou L, Jang J-C: **Sugars as signaling molecules**. *Current Opinion in Plant Biology* 1999, **2**(5):410-418.
16. Udvardi MK, Czechowski T, Scheible W-R: **Eleven golden rules of quantitative RT-PCR**. *The Plant Cell Online* 2008, **20**(7):1736-1737.
17. Voegele RT, Struck C, Hahn M, Mendgen K: **The Role of Haustoria in Sugar Supply during Infection of Broad Bean by the Rust Fungus Uromyces fabae**. *Proceedings of the National Academy of Sciences of the United States of America* 2001, **98**(14):8133-8138.
18. Wahl R, Wippel K, Goos S, Kämper J, Sauer N: **A Novel High-Affinity Sucrose Transporter Is Required for Virulence of the Plant Pathogen Ustilago maydis**. *PLoS Biol* 2010, **8**(2):e1000303.
19. Wieczorke R, Krampe S, Weierstall T, Freidel K, Hollenberg CP, Boles E: **Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in Saccharomyces cerevisiae**. *FEBS Lett* 1999, **464**(3):123-128.
20. Yoder O, Turgeon BG: **Fungal genomics and pathogenicity**. *Current opinion in plant biology* 2001, **4**(4):315-321.