DIFFERENTIAL SUGAR BEET GENE EXPRESSION DURING THE DEFENSE RESPONSE TO CHALLENGE BY *CERCOSPORA BETICOLA*

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Abstract:

Cercospora leaf spot (CLS) caused by the fungus *Cercospora beticola* Sacc. (Saccardo, 1867) (*C. beticola)* is a widespread foliar disease of sugar beet that causes reduced sugar and root yield. It can become a problem in many production areas in the U.S. and world-wide. The study of host resistance is important for the understanding of host-pathogen interaction, the development of more effective disease control strategies, and ultimately marker assisted selection utilizing implicated defense response genes. In the current study, a modified suppressive subtractive hybridization (SSH) was utilized to identify host plant genes involved in the defense response of sugar beet resistant to CLS. A CLS-resistant sugar beet germplasm, (FC504CMS X FC502/2)] X SP6322-0 (LSR), was inoculated with *C. beticola* and a subtracted cDNA library was created to identify defense related genes. Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was used to quantify the expression level of candidate defense response genes over an infection time course. Expression levels were examined for four genes identified via SSH (CP5, P450, PR-10, UVB), plus the sugar beet homologs of two defense response genes known from other systems (GST, SOD).

Introduction and Objectives:

Cercospora Leaf Spot (CLS) caused by *Cercospora beticola* Sacc. (Saccardo 1867) (*C. beticola)* is the most destructive foliar fungal pathogen affecting sugar beet, causing significant sugar losses in most production areas worldwide (Weiland and Koch 2004). While fungicide treatments applied strategically throughout the production cycle can help control CLS (Vereijssen, Schneider, and Jeger 2007), host resistance is rapidly becoming a primary method for control, particularly since the fungus is gaining increased tolerance to commonly used fungicides (Davidson et al. 2006). Qualititative (single gene) and quanitative (multiple genes) plant resistance is an integral part of crop disease management (Jones 2001). Plant defense mechanisms have been extensively studied in model systems (Pegadaraju et al. 2007, Adie et al. 2007) however the underlying basis of the defensive mechanism is poorly understood for most crops. Although this knowledge base provides a platform for drawing hypotheses about resistance in species outside of the model systems, these mechanisms may vary in specific host/pathogen interactions.

It has been estimated that between 4 or 5 major single resistance (*R*) genes are responsible for most resistance to *C. beticola* (Smith and Gaskill 1979, Schafer-Pregl et al. 1999). However, *R* gene (qualitative) resistance is often not durable as pathogen

populations are able to rapidly adapt to overcome individual resistance genes (reviewed by Leach et al. 2001). In many host/pathogen systems quanitative resistance is predicted to be more durable as it is effective against whole groups of pathogens often with multiple gene families contributing to resistance (Van der Plank 1968, Parlevliet 2002). Gaining knowledge of all genetic components in sugar beet that contribute to the control of *C. beticola* will aid in the development of molecular markers for use in incorporating durable resistance into commercial varieties.

Suppressive subtractive hybridization (SSH) is a non-biased approach for examining broad-spectrum changes in gene expression in response to a wide array of stimuli (Diatchenko et al. 1996). SSH amplifies mRNA sequences that are up regulated and suppresses the amplification of sequences that are not differentially expressed. It does this by overcoming sample bias that can occur based on relative differences in abundance in mRNA through a hybridization step that normalizes sequence abundance (Diatchenko et al. 1996). This method has been successfully used for examining the genetic response to plant/pathogen interactions (Kong, Anderson, and Ohm 2005, Lin et al. 2007, Lu et al. 2005) and to uncover contributors to quantitative resistance (Han et al. 2005). Puthoff and Smigocki (2007) recently outlined the utility of SSH for studies in sugar beet, which has limited genomic data available and large varietal variability.

In this work, a modified SSH was utilized to identify host plant genes predicted to be involved in the defense response of a sugar beet hybrid resistant to CLS. A subset of genes with hypothesized roles in defense response were then tested using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) to quantify and verify expression level over time and their role in the defense response to *C. beticola*.

Materials and Methods:

Plant material, fungal culture, and inoculation for SSH. Five plants of a leaf spot resistant (LSR) *Beta vulgaris* L. hybrid (FC504CMS X FC502/2)] X SP6322-0 (Coe and Hogaboam 1971, Smith and Gaskill 1979) were grown in the glasshouse for 8 weeks (16 hour light/8 hour dark; 21°C). Inoculum was prepared from *C. beticola* infected sugar beet leaves (collected from the Sugarbeet Research Unit *C. beticola* disease nursery) following the protocol of Panella (1998). Spore concentration was adjusted to 10^6 per ml with sterile distilled water and this inoculum spray-applied to the adaxial and abaxial surfaces of all fully expanded leaves on each plant. To generate the high humidity environment required for *C. beticola* germination, the plants were bagged for 72 hours prior to inoculation and for an additional 72 hours immediately following the spray inoculation. After the bags were removed, plants remained in the glasshouse for 21 days (16 h light/8 hr dark; 21°C).

RNA isolation for SSH. Approximately 1g/plant of leaf material was collected from five LSR plants at two time points. Tissue for the driver RNA (unchallenged) was collected immediately before *C. beticola* inoculation. Tissue for the tester RNA (challenged) was collected from the same plants 72 hours post inoculation. Total RNA was extracted using the RNeasy Plant Mini kit (Qiagen, Valencia, CA) according to manufacturer's recommendations.

Suppressive Subtractive Hybridization. Equal quantities of tester and driver RNA were used to create cDNA using the Smart cDNA Synthesis system (BD Biosciences, Palo Alto, CA). The SSH protocol was conducted using the PCR-select cDNA subtraction kit (BD Biosciences-Clontech, Mountain View, CA) according to manufacturer's recommendations with slight modification. Briefly, the cDNA was digested with *RsaI* for 3 hours at 37°C. Digested cDNA fragments were recovered and purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). Following adaptor ligation, two hybridizations were performed. In the first hybridization, differentially expressed sequences were equalized and enriched by annealing (8 h at 68°C) and subtracting driver cDNA to and from tester cDNA. In the second hybridization, samples were mixed to further enrich and select for equalized and subtracted cDNA which served as a template for PCR-based differential screening. PCR screening occurred in two stages. First, the equalized, differentially expressed sequences flanked by two different adaptors were amplified exponentially using the adaptor-specific primers. The second PCR amplification further enriched for differentially expressed sequences. A subtracted cDNA library was created by cloning the second round PCR products into a series of pGEM-T vectors (Promega, Madison, WI) that were then transformed into Top10 *E. coli* cells (Invitrogen, Carlsbad, CA). Individual colonies were transferred and grown in LB overnight in replicate 96-well plates.

Sequencing and gene identification. Differentially expressed genes were identified by amplifying the pGEM-T inserts using vector-specific primers (SP6 and T7) using the following PCR conditions: 4 min at 94°C followed by 35 cycles of 94°C (1 min), 42°C (1 min) and 72°C (1 min) and a final extension for 5 min at 72°C. PCR products were sequenced then BLASTX was used to search raw sequence against the GenBank Viridiplantae (all green plants) non-redundant database.

Plant material, fungal culture, and inoculation for RT-PCR. The LSR line, and an additional line, FC403 (Hecker and Lasa 1992), susceptible to *C. beticola*, were grown in a glasshouse for 8 weeks (16 hour light/8 hour dark; 21°C). Inoculum was generated using isolate 7529a provided by Gary Franc (University of Wyoming, Laramie). Mycelial plugs were transferred from potato dextrose agar (PDA) to 50 ml of sterile glucose yeast extract + casein (GYEC) media and incubated at 30°C for 3 days with gentle shaking (150 rpm). Following incubation, the culture was homogenized using a sterile Waring blender and 1 ml evenly spread onto one-half strength V8 agar. After 3-5 days of incubation, the culture was ground in sterile water, filtered through small pore wire mesh and the concentration of mycelial pieces and spores was adjusted to 10^6 per ml. Inoculation of plants was performed as described for the SSH. Sterile water was applied to each genotype as an unchallenged control. Five plants of each genotype were used per treatment with the experiment repeated three times.

RNA isolation for RT-PCR. Three of the five plants were randomly selected from each treatment. Approximately 16 mg of leaf tissue per plant was collected and pooled for each treatment at four time points. Collections were completed prior to inoculation (T_0) then at 48 and 72 hours, and 5 days post *C. beticola* or sterile water inoculation. Tissue was collected in microcentrifuge tubes, flash frozen in liquid nitrogen, and stored at -

80°C. RNA was extracted using the RNeasy Plant Mini kit and was quantified with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).

RT-PCR. Four defense related gene candidates (CP5, P450, PR-10, and UVB) (Table 2) identified in the SSH analysis and two genes that have shown to be related to the defense response in other host systems (Glutathione-S-Transferase (GST) and Superoxide Dismutase (SOD)) (Dudler et al. 1991, Zacheo and Bleve-Zacheo 1988) were further examined by semi-quantitative RT-PCR to assess potential differential expression between resistant and susceptible genotypes during *C. beticola* infection. Primers for RT-PCR were designed from gene sequences generated from the SSH analysis or GenBank accessions (Table 1). Semi-quantitative RT-PCR was performed using a OneStep RT-PCR kit (Qiagen, Valencia, CA) optimized for template concentration and cycle number based upon uniform GAPDH peak intensity for each primer set. PCR products and molecular weight markers were separated by agarose gel electrophoresis and visualized using a BioRad gel documentation system (Bio-Rad Laboratories, Hercules, CA). Peak intensity for all samples was generated using Quantity One Software (Bio-Rad Laboratories, Hercules, CA). Sample peak intensities were normalized by dividing sample peak intensity by the corresponding GAPDH intensity. Ratios of peak intensities over the control GAPDH peak intensity were analyzed for significant changes in gene expression using PROC GLMMIX in SAS (SAS Institute, 1992, Cary, NC).

Table 1. Selected primers for RT-PCR.

Primers	Primer DNA sequence (5' → 3')
P450 FWD	ACC CAT GAT GTT AAA AGA TCA CCT
P450 REV	CCA CTC TAC TGT CAC TGC TGA ACT
CP5 FWD	ACT GCT TTC CAC ATA CCC TGC CTA
CP5 REV	TGA TTG GTC GTC GGA TCT GGG AAT
SOD FWD	TTT CCT CAC CCA CTA CAA GTA CAA
SOD REV	CTC TAC CAA CAA CAG AAT TTG CTC
GST FWD	ATA AGC CAA CTT GGT ACA AGG AAG
GST REV	GGC CTC CCA GAT GTA ATA TCA TAG
UVB FWD	ACA GCA GTT TCG ATG GCT ACT CCA
UVB REV	CAA CAC AGT TCC ACC AGC CAC AAT
PR-10 FWD	ACA TGC ACC CAA GTG TAT GTG GAG
PR-10 REV	GAG GTA CTG GAG ATT TGG GTT GGT
GAPDH FWD	GCT GCT GCT CAC TTG AAG GGT GG
GAPDH REV	CTT CCA CCT CTC CAG TCC TT

Results:

Suppressive subtractive hybridization. A modified forward subtraction using the SSH method was used to identify genes predicted to be up-regulated in a resistant hybrid sugar beet by challenge with *C. beticola.* This allows for discovery of genes that may be involved in a resistant response to the fungus. Approximately 1,700 clones were picked from the subtraction to create the SSH library. A set of clones was randomly selected for sequencing with an average insert size around 350-500 base pairs. Comparison of the

sequenced clones from the SSH library against the non-redundant protein database (nr; NCBI) showed 125 sequences had significant similarity ($\leq e^{-5}$) to existing sequence in the database (Table 2). The putative genes from sugar beet were categorized by biological process according to predicted gene ontology (2). Nine major ontological groups were designated as: primary metabolism; secondary metabolism; cell structure, development and transport; signal transduction; energy; defense and stress related; photosynthesis; oxidative response; and gene expression and protein turnover. Two groups were created for sequences that could not be grouped by ontology, those that lacked significant homology to any proteins in the public databases (unknown protein), or those that matched proteins with unknown molecular function (unknown function) (Table 2). All EST sequences were deposited in GenBank (Table 1). The group with the largest number of genes identified was gene expression and protein turnover (20%), followed by defense and stress response (14%) and primary metabolism (13%). Secondary metabolism (10%), signal transduction (10%) and cell structure, development and transport (6%), were the next abundant groups. Lesser affected categories included, photosynthesis (4%), oxidative response-related (4%), and energy (2%). 14 out of 125 (11%) of the sequences were from unknown proteins and the group with unknown functions contained 7 out of 125 sequences (6%) (Fig. 1).

Table 2. SSH results: *C. beticola* infected CLS resistant germplasm.

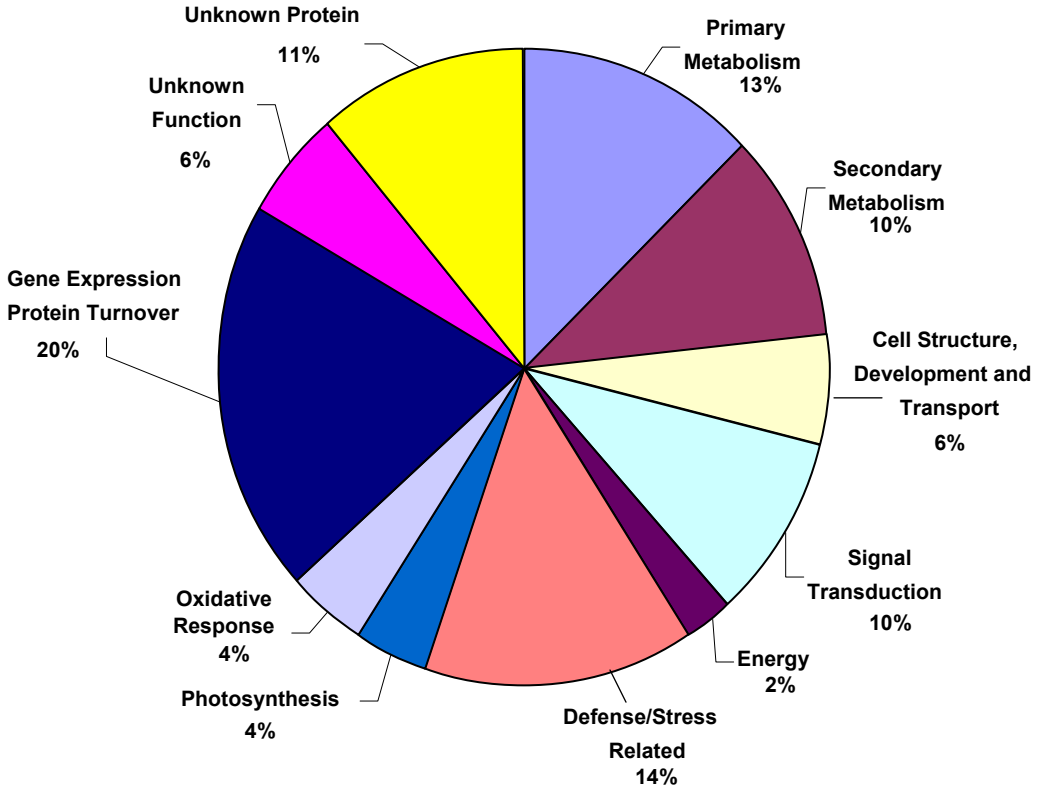
Clone ID	EST GenBank Accession	Times found	Homology	e-value
Primary metabolism				
p2 b6	EX956243	1	short chain dehydrogenase	e-94
p16 c7	EX956352	1	glycerol kinase	e-18
p4 b1	EX956244	1	UDP-glucose:flavonoid-O-glucosyltransferase	e-53
p5 a5	EX956245	1	pyruvate kinase	e-40
p1 a9	EX956359	1	mutase family	e-19
p1 e7	EX956246	1	nucleoside-diphosphate-sugar epimerase	e-50
p16 d1	EX956247	1	dihydroxyacid dehydratase	e-34
p16 c5	EX956248	2	nitrate reductase	e-50
p17 h7	EX956360	1	glyceraldehyde-3-phosphate dehydrogenase A	e-33
p16 d3	EX956249	1	GCN5-related N-acetyltransferase	e-31
P2 F1	EX956250	1	CAX-interacting protein 2	e-45
P2 G9	EX956251	1	allyl alcohol dehydrogenase	e-36
p16 e2	EX956252	1	Rieske [2Fe-2S] region	e-34
p5 c8	EX956253	1	NADH dehydrogenase subunit 1	e-57
p6 h2	EX956254	1	UV-B repressible protein	e-11
p17 h1	EX956255	1	protein phosphatase 2C	e-34
Secondary metabolism				
p6 a1	EX956357	2	S-adenosylmethionine decarboxylase proenzyme 2	e-38
p6 e10	EX956256	1	membrane related protein CP5	e-51
p12 b8	EX956257	1	enoyl-CoA hydratase/isomerase	e-28
p16 b11	EX956258	1	acid phosphatase	e-67
p2 g2	EX956259	1	10-formyltetrahydrofolate synthetase	e-126

p16 h7	EX956260	2	carboxyvinyl-carboxyphosphonate phosphorylmutase	e-72
p1 a7	EX956261	1	magnesium chelatase subunit	e-63
p2 e6	EX956242	2	phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	e-85
p17 B8	EX956262	1	1-aminocyclopropane-1-carboxylate oxidase 1	e-60
p4 e6	EX956263	1	flavanone 3-hydroxylase	e-20
p4 c2	EX956264	1	xanthine permease	e-24
p1 g6	EX956265	1	vitellogenin	e-14
P2 F4	EX956266	1	3-isopropylmalate dehydrogenase	e-100
Cell Structure Development and Transport				
p12 e3	EX956267	1	hypothetical protein	e-28
p1 a11	EX956268	1	cinnamoyl CoA reductase 2	e-39
p4 f1	EX956269	2	glucosyl hydrolase	e-83
p6 c3	EX956270	1	pectin acetylesterase	e-57
p6 h7	EX956271	1	Cwf15/Cwc15	e-68
p17 e10	EX956361	1	nodulin-like protein	e-31
p6 g1	EX956241	1	WAX2	e-45
Signal Transduction				
p16 a3	EX956272	1	hypothetical protein	e-20
p1 c9	EX956273	1	hypothetical protein	e-23
p6 e6	EX956274	1	SPRY domain-containing protein	e-65
p6 b12	EX956358	1	myo-inositol-1-phosphate synthase	e-48
p17 d8	EX956275		GTP binding site protein	e-15
p16 c3	EX956276	1	WD40 domain protein	e-102
p6 h8	EX956277	1	COP1 homolog	e-88
p2 f6	EX956278	1	SPX domain-containing protein	e-57
p5 c4	EX956279	1	serine/threonine protein kinase	e-42
p6 g2	EX956355	1	NBS/LRR resistance -like protein	e-16
p16 g4	EX956280	1	receptor-like protein kinase	e-09
p16 h2	EX956281	1	ABC transporter	e-50
Energy				
p17 g2	EX956282	1	adenylate kinase	e-22
p12 c10	EX956283	1	ATPase	e-44
p2B3	EX956356	4	photosystem II M protein	e-16
Defense/Stress				
p6 b10	EX956284	1	hypothetical protein	e-68
p5 d5	EX956285	1	Putative PR-protein	e-61
p6 f5	EX956286	1	Kil protein (phospholipase C)	e-33
p12 g11	EX956287	1	serine decarboxylase	e-51
p1 g2	EX956288	1	C2 domain-containing protein	e-59
p2 h11	EX956289	1	Drm3	e-14
p17 c4	EX956290	1	PR-10	e-08
p17 g1	EX956291	1	cysteine proteinase	e-24
p2 b7	EX956354	1	lipase class 3 family protein	e-27
p16 a1	EX956292	1	cysteine proteinase	e-20

P2 C10	EX956353	1	cysteine proteinase	E-20
p17 c1	EX956293	1	auxin-associated family protein	e-15
p4 g10-T7	EX956294	1	profilin	e-09
p4 g2	EX956295	1	auxin-induced beta-glucosidase	e-53
p1 g8	EX956296	1	salt tolerance protein	e-18
p6 h1	EX956297	1	lipid binding protein	e-08
p6 e7	EX956298	1	auxin-induced SAUR-like protein	e-26
p12 c2	EX956299	1	jacalin lectin	e-12
Photosynthesis				
p1 d3	EX956300	1	photosystem I reaction center subunit N	e-13
p12 d1	EX956301	3	photosystem I p700 apoprotein A2	e-73
P17 B11	EX956302	3	RuBisCO	e-29
p6 h11	EX956303	2	chloroplast PSI type III chrolophyll a/b-binding protein	e-47
p17 h8	EX956304	1	phytoene synthase	e-59
Oxidative Response				
p4 e5	EX956305	4	Thioredoxin	e-57
p1 c6	EX956306	1	ubiquinone oxidoreductase	e-33
p4 f2	EX956307	1	NADPH-cytochrome P450 reductase	e-82
nox	EX956239	1	nitric oxide oxidase 2	e-53
p1 d4	EX956363	1	catalase 2	e-60
Unknown Function				
p2 c6	EX956308	1	ycf1	e-25
p17 d3	EX956309	2	amine oxidase	e-65
P2 C1	EX956310	1	flowering locus T-like protein	e-30
P2 F7	EX956311	4	A1-induced protein	e-40
p2 e12	EX956312	2	zinc finger B-box protein	e-39
p2 H12	EX956313	1	Geranylated protein ATGP4	e-37
p17 g6	EX956314	1	CLC-d chloride channel protein	e-94
Unknown Protein				
p6 a5	EX956315	1	hypothetical protein	e-17
p6 e12	EX956316	2	hypothetical protein	e-41
p17 c5	EX956317	1	hypothetical protein	e-62
p16 f7	EX956318	1	hypothetical protein	e-24
p16 a2	EX956319	1	hypothetical protein	e-38
p2 a10	EX956320	1	hypothetical protein	e-24
p17 c10	EX956321	2	hypothetical protein	e-180
p5 d4	EX956322	1	hypothetical protein	e-13
p1 c5	EX956323	1	hypothetical protein	e-12
p12 c8	EX956324	2	hypothetical protein	e-19
p1 d8	EX956325	1	hypothetical protein	e-68
p6 d1	EX956326	1	hypothetical protein	e-26
p17 e1	EX956327	1	hypothetical protein	e-27
p5 f2	EX956328	1	hypothetical protein	e-53
Gene Expression/Protein Turnover				

p17 d4	EX956329	1	hypothetical protein	e-14
p6 g9	EX956330	1	hypothetical protein	e-27
p4 b2	EX956331	1	YABBY protein	e-53
p1 f9	EX956332	1	Der1-like protein	e-19
p4 c1-T7	EX956333	1	translation initiation factor	e-31
p1 e10-T7	EX956334	1	Polyubiquitin	e-49
p17 d4	EX956329	1	bZIP transcription factor	e-28
p17 c11	EX956335	1	ribosomal protein S2	e-78
p1 f2	EX956336	1	ubiquitin conjugating enzyme	e-43
p1 f3	EX956337	1	ubiquitin	e-68
p17 b2	EX956338	1	membrane-anchored ubiquitin-fold protein 3	e-28
p1 f5	EX956339	1	zinc finger domain	e-15
p1 f7	EX956341	1	helix-hairpin-helix motif, class 2	e-08
p4 h9	EX956340	1	RNA polymerase	e-31
P2 D2	EX956342	1	60S ribosomal protein 110a-1	e-50
p16 h4	EX956343	1	leucine zipper transcription factor	e-21
p6 a2	EX956344	1	peptide chain release factor subunit-1-3-like protein	e-70
p6 b1	EX956345	1	ubiquitin-conjugating enzyme E2	e-51
p16 d5	EX956346	1	DnaJ protein	e-30
p5 b5	EX956240	1	30S ribosomal protein	e-49
p17 f11	EX956347	1	ATHB-12 homeobox-leucine zipper protein	e-21
p17 b10	EX956348	1	microtubule associated protein	e-30
p5 g9	EX956349	1	ethylene-responsive transcriptional coactivator	e-21
p17 g10	EX956350	1	ubiquitin-conjugating enzyme family protein	e-18
p17 c3	EX956351	1	ubiquitin conjugating enzyme	e-60

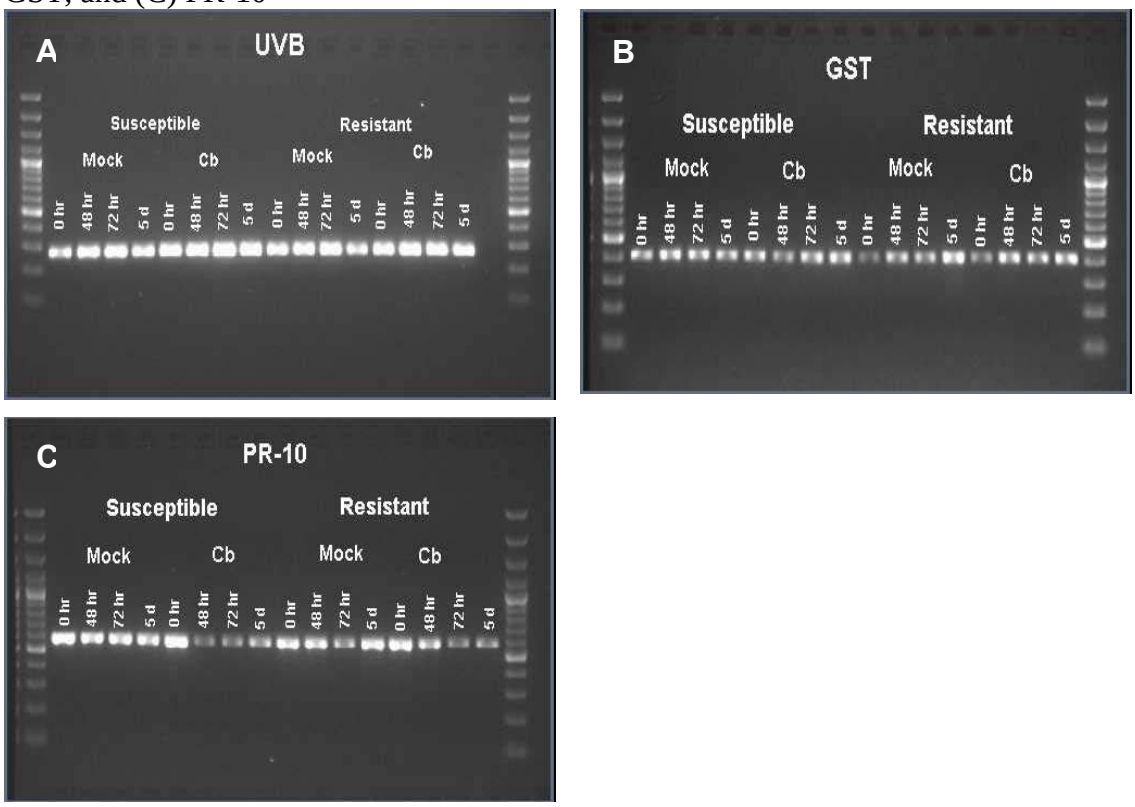
Figure 1. Classification of putative genes implicated in the interaction between unchallenged and *C. beticola*-challenged resistant sugar beet germplasm based on gene sequence homology.



Differential gene expression of select candidate genes using semi-quantitative RT-PCR. To validate the specificity of certain transcripts that contribute to resistance to *C. beticola*, individual genes were further studied to confirm differential gene expression in a susceptible and resistant interaction. Six genes that have been reported to be associated with the plant defense response or were identified in the SSH, were selected (CP5, GST, P450, SOD, UVB, and PR-10) for further studies by semi-quantitative RT-PCR. CP5, P450, and SOD had no significant difference in gene expression levels ($p=0.05$) in either the susceptible or resistant hosts challenged and unchallenged with *C. beticola.* UVB had different levels of gene expression at different time points (p-value 0.0133). It was up-regulated early in the defense response and then significantly down-regulated 5 days after inoculation (Fig. 2A). GST was significantly down-regulated in the resistant host (p-value 0.0380) with the highest expression levels occurring at 48 and 72 hours after inoculation (Fig. 2B). Interestingly PR-10 was slightly down-regulated in both susceptible and resistant plants after inoculation as compared to the mock treatment (p-value 0.0570) (Fig. 2C). This suggests that PR-10 may be involved in a general plant defense pathway. In general the RT-PCR did not give substantial information regarding contribution of the selected genes to resistance against *C. beticola.* Because the SSH was completed only on the resistant hybrid, it is not possible to know whether the genes identified contribute only to the resistant interaction. They may be involved in a more general response to *C. beticola* infection that is also exhibited by the susceptible line.

Further subtractive analysis should be completed additionally using a susceptible line, to identify those sequences that only contribute to the resistant response during *C. beticola* challenge.

Figure 2. Verification of specific products from RT-PCR reactions for (A) UVB, (B) GST, and (C) PR-10



Discussion:

A modified forward suppressive subtractive hybridization cDNA library was created to identify genes potentially up-regulated by *C. beticola* in a resistant hybrid sugar beet. Several biological processes were affected by pathogen challenge, including carbohydrate metabolism, glycolysis, nitrogen assimilation, lipid metabolism, biosynthesis of secondary metabolites and hormone production. Induction of these sequences is consistent with other studies of gene expression in host-microbe interactions (Balaji, Gibly, and Debbie 2007) and is indicative of a shift in metabolic processes towards synthesis of defense-related compounds (Shigaki and Bhattacharyya 2000). Of all the major ontological categories identified, of particular interest were the defense and stress related genes induced by *C. beticola.* The types of genes induced provide a platform for formulating hypotheses about the important players in the defense mechanism, including perception of the pathogen, signal transduction cascades responsible for activation of resistance mechanisms, changes to physical barriers, antioxidative deterrents against the pathogen, and activation of systemic resistance.

There are a diverse array of defense mechanisms that exist in plants, including innate defenses, such as physical barriers and constitutively expressed antimicrobial

compounds (Zhao et al. 2005), and inducible resistance mechanisms, such as the hypersensitive response (Kryzmowska et al. 2007), the oxidative burst (Lee and Hwang 2005) and systemic resistance (Park et al. 2007). Activation of many of these defense constituents requires an effective pathogen recognition system. The most well known pathogen perception components consist of major single resistance (*R)* genes (Hulbert et al. 2001, Hammond-Kosack and Jones 1997). Several *R* gene classes were induced in LSR by *C. beticola* (serine threonine-kinase, p5 c4; NBS/LRR, p17, g5; receptor-like protein kinase, p16, g4) as well as some components targeted by *R* genes such as inositol phosphate synthase (p6, b12) (Hammond-Kosack and Jones 1997, Rinaldi et al. 2007).

Following recognition of the pathogen, several signal transduction cascades can be activated, involving secondary signal molecules such as salicylic acid, jasmonic acid, and ethylene (reviewed in Zhao and Qi 2008). Signal transduction-related constituents constituted 10% of the total genes identified in our study. Some of the genes predicted to be up-regulated by *C. beticola* in sugar beet suggest a potential role for the octadecanoid pathway and ethylene signaling in resistance activation.

Further evidence for a general defense mechanism during the *C. beticola* resistance response includes the identification of secondary metabolism-related proteins related to sterol binding (CP5) and biosynthesis (acid phosphatase). This suggests a role for antimicrobial glycosides in protection against pathogen attack in plants (Simons et al. 2006). Furthermore, a cinnamoyl CoA reductase was identified, which has been shown to act in conjunction with G-protein signaling (involved in physical barrier strengthening through lignin synthesis) and is induced in rice following resistance activation (Kawasaki et al. 2006). Additional evidence for cell wall modification in response to *C. beticola* challenge include the up regulation of a putative glucosyl hydrolase (cellulose degradation) and a pectin acetyltransferase.

Systemic resistance in plants initially includes an oxidative burst, followed by a hypersensitive response and elicitation of a series of defense-related proteins, most notably pathogenesis-related (PR) proteins (de Wit 2007). Although there were oxidative enzymes induced by *C. beticola* in LSR, they are primarily detoxifying proteins (GST, thioredoxin, catalase). An oxidative burst leading to hypersensitive response would not be expected in sugar beet in response to this pathogen, which has a necrotrophic phase that would benefit from cell death and not limit its rate of growth (Govrin and Levine 2000). *Cercospora* is one of eight genera of fungi which produce nonspecific toxins (e.g. cercosporin). Cercosporin is a key component of the necrotrophic phase of pathogenesis for this pathogen, and uses light energy to react with molecular oxygen to generate an array of oxygen free radicals (Daub and Erenshaft 2000). The importance of antioxidants in *C. beticola* disease control has been demonstrated by conferring increased resistance to the fungal pathogen through transgenic over-expression of superoxide dismutase (Tertivanidis et al. 2004) and induction of catalases in corn (Williamson and Scandalios 1992).

Several classes of pathogenesis-related genes, indicative of systemic resistance, were induced by *C. beticola* in LSR. These include a putative PR-protein, PR-10, cysteine proteinase, beta-1, 3-glucanase, and a lipid binding protein. PR-10 has ribonuclease function, is induced in other plant systems by fungal pathogens (Lo, Hipskind, and Nicholson 1999), and in rice blast, early and more elevated expression of PR-10 leads to greater disease control of *Magnaporthe grisea* (Kim et al. 2004).

However, using the semi-quantitative RT-PCR, PR-10 was down-regulated during the resistant response.

While suppressive subtractive hybridization (SSH) revealed several differentially-expressed proteins, RT-PCR studies on a select few of those genes were unable to clarify the role they might play in the sugar beet defense response to *C. beticola*. Additional work needs to be done to determine if individual proteins are indeed quantitatively and differentially expressed throughout several time points during infection and how they individually and collectively contribute to *C. beticola* resistance. This analysis provides valuable information on potential players in the defense response and can help focus future research on particular genes and their contribution to plant resistance.

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