# The Cell Wall Integrity-Associated Map Kinase Homolog, *AbSlt2* in the Necrotrophic fungus *Alternaria brassicicola* is Required for Pathogenicity of Brassicas

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## ABSTRACT

Using the genome database of the phytopathogenic fungus, *Alternaria brassicicola*, we identified a gene with high homology to the cell wall integrity-associated mitogen-activated protein (MAP) kinase, *Slt*2 in the yeast, *Saccharomyces cerevisiae*. This MAP kinase consists of a predicted 1,251-bp open reading frame, and encodes a 416-amino-acid protein weighing 47501 Da. This homolog was designated *AbSlt2* (*A. brassicicola Slt2*) and gene disruption knockout (KO) mutants were generated in an *A. brassicicola* wild type background. Several altered phenotypes were found in the mutants compared to the wild type. During growth in various liquid and solid media, the *abslt2* mutants displayed slightly aberrant hyphal growth and were unable to develop at the same rate as wild type. Furthermore, scanning electron microscopy (SEM) analysis revealed the *abslt2* mutants showed decreased penetration ability, underdeveloped appresoria, and altered morphology on the leaf surface of the host plant, *Brassica oleracea* (cabbage) when compared to wild type. *Abslt2* mutant hyphae exhibited slower growth *in planta* ultimately resulting in highly reduced virulence. Complementation of the disruption mutant with the wild

mutant with the wild type gene fully restored pathogenicity. Therefore, *AbSlt2* is a new pathogenicity and developmental factor in *A. brassicicola.*

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## INTRODUCTION

*Alternaria* is an extremely diverse genus of the phylum Ascomycota consisting of both saprophytic and pathogenic species. Found on plants and in food, soil, and air, *Alternaria* spp. have emerged as opportunistic pathogens of plants and humans, particularly in patients with immunosuppression (Morrison et al, 1993.) Cases of onychomycosis, sinusitis, ulcerated cutaneous infections, and keratitis, as well as visceral infections and osteomyelitis due to *Alternaria* have been reported (Anaissie et al 1989, Garau et al 1977, Manning et al 1991, Schell, 2000).

Among the most destructive plant diseases, the so-called "rots" are caused by necrotrophic fungi that inflict substantial tissue damage on hosts in advance of hyphal colonization. *A. brassicicola* causes black spot disease of cruciferous plants and is of worldwide economic importance in cultivated Brassica species such as canola, cabbage, and mustards (Lawrence, 2003). Infections by this parasite have been reported from many countries including Australia (Sivapalan & Browning, 1992), Canada (Petrie, 1974), South Africa (Holtzhausen & Knox-Davies, 1974), Taiwan (Wu, 1979), the UK (Maude & Humpherson Jones, 1980) and the USA (Babadoost & Gabrielson, 1979). Black spot can cause considerable reduction in the quality of harvested Brassica products, and no known source of resistance has been identified among Brassica crop species (Sigareva & Earle, 1999). No satisfactory resistance has been reported in cultivated species, however, high levels of resistance to this fungus have been reported in weedy cruciferous plants including the model plant, *Arabidopsis thaliana*. Direct losses plus the cost of control strategies contribute to the huge agricultural significance of this organism and the genus overall.

## MAP KINASE PATHWAYS IN FUNGI

Fungi are eukaryotic organisms with a well-defined, rigid, polysaccharide-based cell wall. They are chemoheterotrophs (organisms that require organic compounds for both carbon and energy sources) and obtain their nutrients by absorption. They are typically classified as saprophytes (organisms that live off of decaying matter) or parasites (organisms that live off of living matter) or often a combination. As with the bacteria, fungal virulence factors can be divided into two categories: virulence factors that promote fungal colonization of the host; and virulence factors that damage the host or suppress defense. We decided to investigate the fungus, *A. brassicicola* by initially searching for and analyzing genes and gene products that alter its ability to colonize the host. We identified an *A. brassicicola* mitogen-activated protein (MAP) kinase gene, *AbSlt2,* a homolog of *Saccharomyces cerevisiae Slt2/Mpk1*that is required for proper cell shape and integrity (Hahn and Thiel, 2002). Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens) and regulate various cellular activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis (Pearson *et al*, 2001).

In virtually all organisms, MAP kinases are integral parts of gene cascades generally consisting of three conserved phosphokinases, MAP kinase kinase kinases (MAPKKKs), MAP kinase kinases (MAPKKs), and MAP kinases (MAPKs), that sequentially activate and mediate appropriate cellular responses to specific environmental stimuli (Blumer and Johnson 1994). MAPKs are involved in the action of most non-nuclear oncogenes in mammals. They are responsible for cell response to growth factors such as BDNF or nerve growth factor for example. Extracellular stimuli

lead to activation of a MAP kinase via a signaling cascade ("MAPK cascade") composed of MAP kinase, MAP kinase kinase (MapKK), and MAP kinase kinase kinase (MapKKK). A MAPKKK that is activated by extracellular stimuli typically phosphorylates a MAPKK on its serine and threonine residues, and then MAPK pathways can be recruited by a wide variety of stimuli including hormones (e.g. insulin and growth hormone), mitogens (e.g. epidermal growth factor and platelet-derived growth factor) vasoactive peptides (e.g. angiotensin-II and endothelin), inflammatory cytokines of the tumor necrosis factor (TNF) family and environmental stresses such as osmotic shock, ionizing radiation and ischemeic injury.

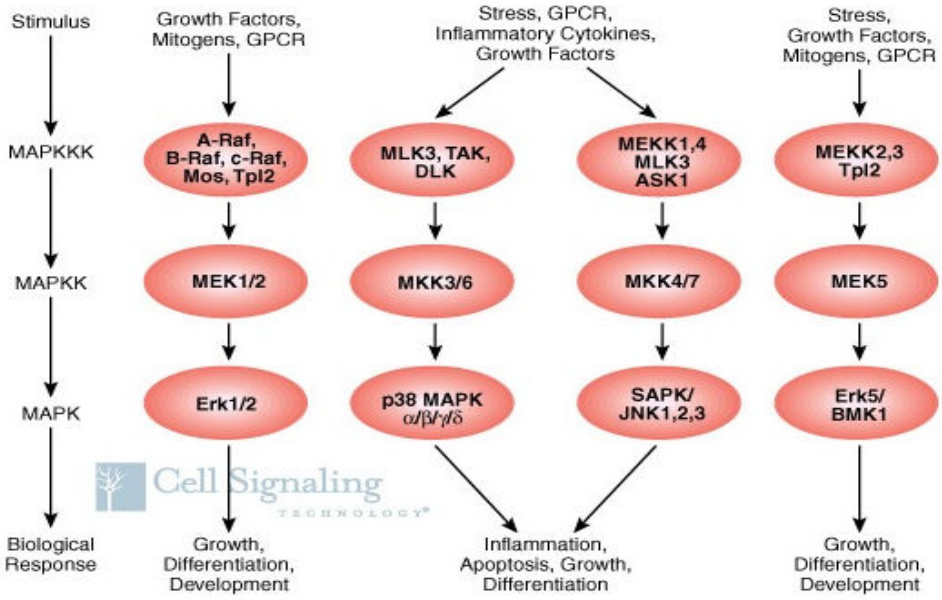



**FIG 1: Mitogen-Activated Protein Kinase Cascades**

**(http://www.cellsignal.com/reference/pathway/MAPK_Cascades.html)**

Fungal pathogens of plants have developed many mechanisms to evade or overcome healthy host plant defenses. Although plants do not have a typical immune system as found in animals, their cells have a thick, complex wall that acts as a barrier to

invasion. Plants display innate pathogen-specific resistance, genetically controlled via resistance genes. Additionally, plants display inducible systemic acquired resistance, which occurs when previous exposure to a pathogen activates signaling pathways acting via molecules such as jasmonate, ethylene, and salicylic acid. These small and sometimes volatile molecules spread throughout the plant or even the plant population. This triggers responses such as the expression of "pathogenesis-related proteins," including antifungal chitinases or glucanases, which can lead to the increased resistance of the whole plant against a subsequent pathogen attack (Pieterse and Van Loon, 2004). Commonalities of the defense systems of different hosts against fungal pathogens include programmed cell death and oxidative burst response (Mur *et al.,* 2006 & Nascimento *et al.,* 2002). Plant pathogenic microbes (fungal and bacterial) release or present molecules considered to be pathogenicity associated molecular patterns (PAMPs). Fungus-derived polysaccharides and proteins are recognized (usually indirectly) by conserved receptors in animals and plants and elicit a defense response. These receptors are often transmembrane proteins with leucine-rich repeat domains and are manifested as resistance gene products in plants and Toll-interleukin (TIR) receptors in animal and insect cells (Belkhadir *et al.,* 2004). This appears to be either an ancient conserved eukaryotic pathway (Nurnberger *et al.,* 2004) or the result of convergent evolution whereby similar motifs have been recruited for defense in different systems (Ausubel, 2005).

Many of these plant and pathogen processes are made possible through the activity of MAPK pathways. In the budding yeast, *Saccharomyces cerevisiae,* five conserved MAP kinase signal transduction pathways have been extensively studied (Banuett, 1998; Gustin *et al.,* 1998; Herskowitz, 1995). Knowledge regarding MAP

kinases and associated pathways in yeast has been instrumental to our understanding of MAP kinases in other eukaryotes, particularly other fungi. In numerous published reports, MAP kinases were shown to be important for pathogenesis in many fungal pathogens, including *Alternaria brassicicola* (Cho *et al*., 2007), *Botrytis cinerea* (Zheng *et al.,* 2000), *Candida albicans* (Alonso- Monge *et al.,* 1999; Csank *et al.,* 1998; Navarro-Garcia *et al.,* 1995), *Cochliobolus heterostrophus* (Lev *et al.,* 1999), *Colletotrichum lagenarium* (Takano *et al.,* 2000), *Fusarium oxysporum* (Di Pietro *et al.,* 2000), *Magnaporthe grisea* (Xu and Hamer, 1996; Xu *et al.,* 1998), and *Ustilago maydis* (Mayorga and Gold, 1999; Muller *et al.,* 1999).

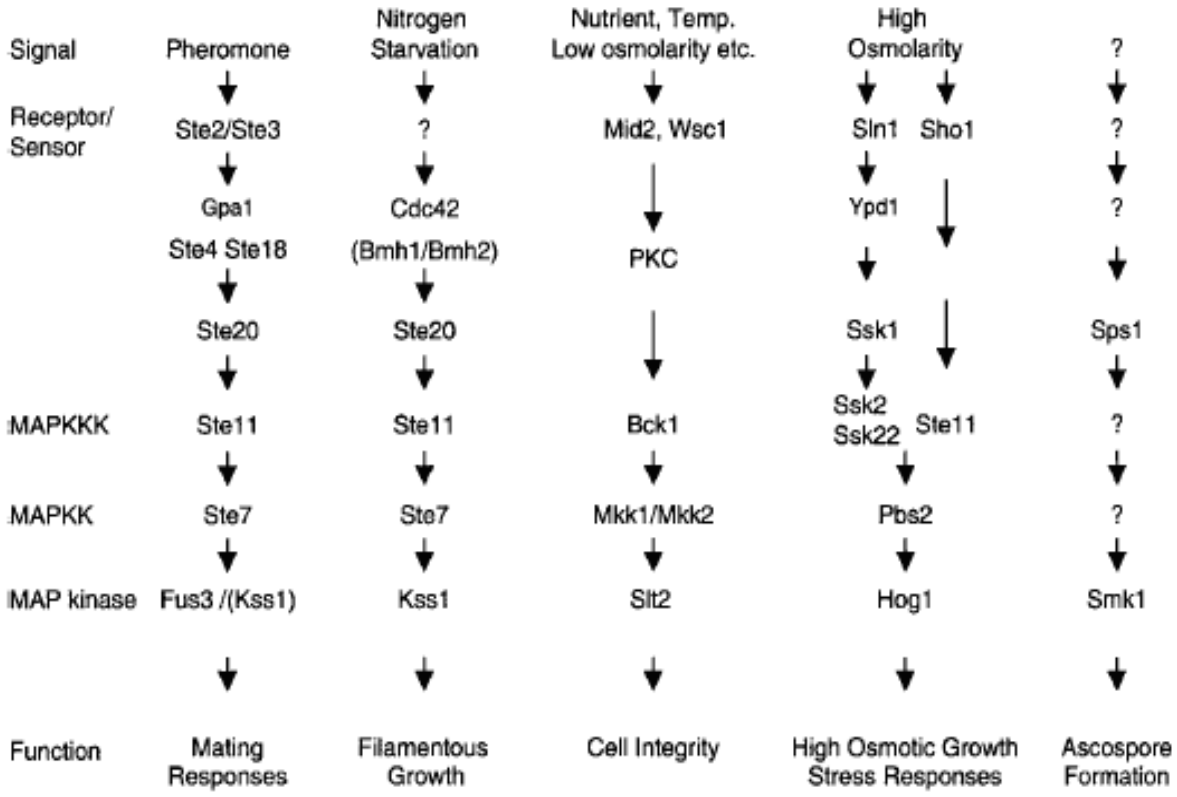



**FIG 2: Five *Saccharomyces cerevisiae* MAP kinase pathways. The MAPKKK, MAPKK, and MAP kinase cascades regulating mating responses, filamentation, cell integrity, and high osmolarity growth function in vegetative cells grown under different conditions (adapted from Xu, 2001).**

The five MAP kinase signal transduction pathways in yeast are associated with regulation of mating, filamentous growth, high osmolarity responses, maintenance of cellular integrity, and ascospore formation. All five of these categories are of importance to the life cycle of yeast as well as many fungi, but the pathway maintaining cellular integrity is an attractive target for disruption for a simple reason. The cell wall serves a similar purpose in all organisms that possess them. The wall gives cells rigidity and strength, offering protection against mechanical and environmental stress. In multicellular organisms, it permits the organism to build and hold its shape. The cell wall also limits the entry of large molecules that may be toxic to the cell. It further permits the creation of a stable osmotic environment by preventing osmotic lysis and helping to retain water. The composition, properties, and form of the cell wall may change during the cell cycle and depend on growth conditions.

Not all species of fungi have cell walls but in those that do, the plasma membrane is surrounded by three layers of cell wall material. From inside out these are: a chitin layer (polymer consisting mainly of unbranched chains of N-acetyl-D-glucosamine) a layer of β-1,3-glucan, and a layer of mannoproteins (mannose-containing glycoproteins) which are heavily glycosylated at the outside of the cell. The most significant components of the fungal cell wall are the polysaccharides chitin and β-1,3-glucan. These components are absent in higher eukaryotes and, therefore, the fungal cell wall is an attractive target for designing specific antifungal agents with selective toxicity (Mehrabi et al., 2006).

The Pkc1p kinase in yeast controls a highly-conserved cell wall integrity signaling pathway that regulates functions essential for growth and the integrity of

proliferating cells (Lee, *et al.,* 1993, Sussman, *et al.,* 2004, & Martin *et al.,* 1996). This pathway consists of a cascade of phosphorylation reactions initiated with the activation of Pkc1p (Levin *et al.,* 1990 & Paravicini *et al.,* 1992). Pkc1p then activates a basic three-protein kinase module involving an integration of the MEK-kinase Bck1p (Costigan C, *et al.,*1994 & Lee and Levin, 1992**)**, the redundant MEK-kinases Mkk1p and Mkk2p (Irie, *et al.,* 1993), and the MAP kinase Slt2 (Torres, *et al.,* 1991). This Slt2 MAP kinase pathway positively regulates growth and cell proliferation and responds to different signals, including low osmolarity, high temperature, mating pheromones, and nutrient limitations (Zarzov *et al.,* 1996). Yeast *slt2* mutants are sensitive to elevated temperatures, caffeine, and cell wall-degrading enzymes and are defective for growth in low-osmolarity and nutrient-poor media (Lee *et al.,* 1993). The *MKC1* gene is a homologue of the *S. cerevisiae SLT2* MAP kinase. Mutants deleted of the *MKC1* gene are viable, but growth rate and cell viability are significantly reduced in cultures grown at 42 C (Navarro-Garcia *et al.,*1995). These defects can be remedied by supplying osmotic stabilizers, such as 1 M sorbitol, in the medium. In an experimental infection system using a murine model and the opportunistic yeast pathogen, *C. albicans*, a homozygous *mkc1* deletion mutant was found to be less virulent than the parental strain (Diez-Orejas *et al.,* 1997), proving that the cell integrity pathway is involved in the pathogenesis. MPS1 (MAP kinase for penetration and sporulation), the homologue of Slt2 in the rice blast fungus *Magnaporthe grisea,* is important for comparison because it belongs to the same phylum (Ascomycota) as the phytopathogenic fungus *A. brassicicola.* Gene replacement mutants of *MPS1* in *M. grisea* were found to be nonpathogenic on rice leaves (Xu *et al*., 1998). In contrast to *pmk1* mutants, *mps1* mutants are capable of

infecting rice plants through wound sites and form melanized appresoria on Teflon membranes or hydrophilic surfaces with the addition of 10 mM cAMP. However, appresoria formed by *mps1* mutants failed to penetrate underlying plant cells and failed to form infectious hyphae (Xu *et al.,* 1998). Therefore, *MPS1* is essential for appresorial penetration.

| Fungal pathogen | MAPK | Major functions |
|---|---|---|
| *M. grisea* | Mps1 | Pathogenicity, penetration, conidiation, cell wall integrity |
| *C. lagenarium* | Maf1 | Pathogenicity, appressorium formation, conidiation |
| *C. purpurea* | Cpmk2 | Pathogenicity, penetration, conidiation, cell wall integrity |
| *F. graminearum* | Mgv1 | Pathogenicity, hyphal fusion, cell wall integrity |
| *M. graminicola* | MgSlt2 | Pathogenicity, infectious growth |
| *B. cinerea* | Bmp3 | Pathogenicity, penetration, macro- and microconidiation |
| *C. albicans* | Mkc1 | Virulence, cell wall biogenesis, stress response |
| *C. neoformans* | Mpk1 | Virulence, cell wall biogenesis, stress response |

**FIG 3: Slt2 homologs in pathogenic fungi**

Slt2 and its homologs have been described in detail across many different phytopathogenic fungi. The following results help elucidate its role in the Brassica pathogen *A. brassicicola*.

## ALTERNARIA BRASSICICOLA PATHOGENESIS

*A. brassicicola* causes black spot disease and can affect Brassica host species at all stages of growth and development, including seeds. On seedlings symptoms include dark stem lesions immediately after germination that can result in damping-off, or stunted seedlings. When older plants become infected, Alternaria symptoms often occur on the older leaves, since they are closer to the soil and are more readily infected as a consequence of rain splash or windblown rain. Late infection, or infection of older leaves,

does not characteristically reduce yields, and can be controlled through intensive removal of infected leaves (Chupp and Sherf, 1960). *A. brassicicola* sporulates in a temperature range of 8 to 30 C, where mature spores occur after 43 and 14 hours respectively. Optimum temperatures are between 18 and 30 C where the average sporulation time is 13 hours. Moisture in the presence of rain, dew, or high humidity is essential for infection, and a minimum of 9-18 hours is required for the species (Humperson-Jones, 1989). Continuous moisture of 24 hours or longer practically guarantees infection (Chupp and Sherf, 1960; Rangel, 1945). Relative humidity of 91.5%
(at 20 C) or higher will result in the production of large numbers of mature spores in 24 hours (Humpherson-Jones and Phelps, 1989).

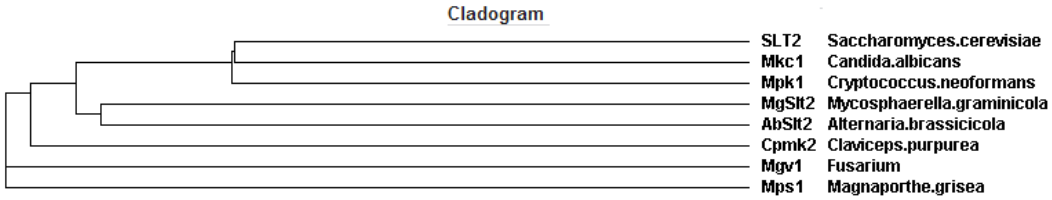
Generally, blackspot disease occurs when *A. brassicicola* airborne conidia land on the host leaf surface. After spore attachment, a conidium germinates and an appresorium develops from the end of a fungal germ tube. It then grows into the underlying epidermal cells using a penetration peg. Once in the plant, the fungus spreads through the plant, and typical necrotic lesions develop on the surface of host leaves. Eventually, aerial conidiophores differentiate from hyphae within the lesions, releasing thousands of new conidia. These conidia are easily dispersed, providing inoculum for the next infection cycle. The most important part of this process is when the conidia land on the host surface. Since Alternaria is a necrotroph, it must kill the host plant's cells to survive. This is accomplished by secretion of toxins consisting of secondary metabolites and proteins. These toxins cause necrosis of tissue, programmed cell death, and are thought to prevent plant defenses from functioning properly. Plants do, however, in some select cases, have evolved mechanisms of defending themselves against these toxins (Agrios, 1997).

## RESULTS

### *AbSlt2* is a homolog of the *S. cerevisiae* MAP kinase *Slt2.*

Using the genome sequence and corresponding annotation of *Alternaria brassicicola,* we identified a predicted protein with 67% identity and 80% similarity at the amino acid level with that of the yeast *Slt2* gene. Briefly, the yeast *Slt2* gene sequence was used to first perform Blastn-based homology search of databases containing the whole genome assembly and the predicted open reading frames. Using these results along with the predicted protein sequence, the *AbSlt2* (*Alternaria brassicicola Slt2* homolog) possesses a 1,251-bp open reading frame encoding a 416-amino-acid sequence with a calculated molecular weight of 47.5 kDa. The protein contains all conserved protein kinase subdomains, including the MAP kinase dual phosphorylation sites TEY (boxed region).

A




B

```
SLT2            MADK-----IERHTFKVFNQDFSVDKRFQLIKEIGHGAYGIVCSARF------------ 42
Mkc1            MDQQEAPIYYGRSVNKVYNQEFIIDSRFKIVKELGHGAYGIVCSAKYDNGSKKVPDSNNG 60
Mgv1            MGDLQ-----GRKVFKVFNQDFVVDERYTVTKELGQGAYGIVCAAVN------------ 42
Mps1            MSDLQ-----GRKIFKVFNQDFIVDERYTVTKELGQGAYGIVCAAVN------------ 42
Cpmk2           MTDIP-----GRRAFKCFNQEFVVDERYTVTKELGQGAYGIVCAAVN------------ 42
MgSlt2          MGDLQ-----GRKVFKVFNQDFIVDERYTVTKELGQGAYGIVCAATN------------ 42
AbSlt2          MGDLA-----NRKVFKVFNQEFIVDERYNVTKELGQGAYGIVCAATN------------ 42
Mpk1            MDNTP------RHLFQTPNNVYILQQPWQFVKELGQGAYGCVSSARN------------ 41
                * :        *   :  *: : ::. : . **:*:**** *.:*
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```
SLT2            --AEAAEDTTVAIKKVTNVFSKTLLCKRSLRELKLLRHFRGHKNITCLYDMDIVFYPD-- 98
Mkc1            NASSSANASFVAIKKITNIFSKNILCKRALRELKLLQFFRGHKNITCLYDLDIIPNPMT- 119
Mgv1            ----NQTNEGVAIKKVTNVFSKKILAKRALREIKLLQHFRGHRNITCLYDMDIP-RPD-- 95
Mps1            ----NQTSEGVAIKKVTNVFSKKILAKRALREIKLLQHFRGHRNITCLYDMDIP-RPD-- 95
Cpmk2           ----NQTKESVAVKKVTNVFSKKILAKRALREIKLLQHFRGHRNITCLYDMDIP-RPD-- 95
MgSlt2          ----NQTGEGVAIKKVTNVFSKKILAKRALREIKLLQHFRGHRNITCLYDMDIP-RPD-- 95
AbSlt2          ----NQTGEGVAIKKVTNVFSKKILAKRALREIKLLQHFRGHRNITCLYDMDIP-RPD-- 95
Mpk1            ----SSTGETCAVKKVTNVFQKKILTKRCLRELRLLHHFRGHKNITCLYDMDIVFDPPGS 97
                          *:**:**:*.*.:* **.***::**:.****:*******:**   *

SLT2            GSINGLYLYEELMECDMHQIIKSGQPLTDAHYQSFTYQILCGLKYIHSADVLHRDLKPGN 158
Mkc1            GEFNEIYLYEELMECDMHQIIRSGQPLSDQHYQSFIYQVLCGLNFIHSADVLHRDLKPGN 179
Mgv1            -NFNETYLYEELMECDLAAIIRSGQPLTDAHFQSFIYQILCGLKYIHSANVLHRDLKPGN 154
Mps1            -NFNETYLYEELMECDLAAIIRSGQPLTDAHFQSFIYQILCGLKYIHSANVLHRDLKPGN 154
Cpmk2           -VFNETYLYEELMECDLAAIIRSGQPLTDAHFQSFIYQILSGLKYIHSANVLHRDLKPGN 154
MgSlt2          -NFNETYLYEELMECDLAAIIRSGQPLTDAHFQSFIYQILCGLKYIHSANVLHRDLKPGN 154
AbSlt2          -NFNECYLYEELMECDLAAIIRSGQPLTDAHFQSFIYQILCGLKYIHSANVLHRDLKPGN 154
Mpk1            GQFREVYLYEELMEADLHAIIRSGQPLSDAHFQSFLYQTLCGLKYIHSANVLHRDLKPGN 157
                  :.  ********.*:  **:*****:* *:*** ** *.**::****:**********

SLT2            LLVNADCQLKICDFGLARGYSENPVEN----SQFLTEYVATRWYRAPEIMLSYQGYTKAI 214
Mkc1            LLVNADCELKICDFGLARGFSENPDEN----AGFMTEYVATRWYRAPEIMLSFTNYTKAI 235
Mgv1            LLVNADCELKICDFGLARGFSVDPEEN----AGYMTEYVATRWYRAPEIMLSFQSYTKAI 210
Mps1            LLVNADCELKICDFGLARGFSVDPEEN----AGYMTEYVATRWYRAPEIMLSFQSYTKAI 210
Cpmk2           LLVNADCELKICDFGLARGFSADPEQN----AGYMTEYVATRWYRAPEIMLSFQSYTKAI 210
MgSlt2          LLVNADCELKICDFGLARGFSVDPEEN----AGYMTEYVATRWYRAPEIMLSFQSYTKAI 210
AbSlt2          LLVNADCELKICDFGLARGFSMDPEEN----AGYMTEYVATRWYRAPEIMLSFQSYTKAI 210
Mpk1            LLVNADCELKICDFGLARGFQPGAVQTDQGQAGFMTEYVATRWYRAPEIMLSFANYTSSI 217
                *******:***********:. .. :.    : ::*****************: .**.:*

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Mkc1            DIWSVGCILAELLGGKPLFRGKDYVDQLNQILMILGTPPESTLQRIGSHRAQNYVRSLPI 295
Mgv1            DVWSVGCILAELLGGRPFFKGRDYVDQLNQILHILGTPNEETLSRIGSPRAQEYVRNLPF 270
Mps1            DVWSVGCILAELLGGRPFFKGRDYVDQLNQILHILGTPNEETLSRIGSPRAQEYVRNLPF 270
Cpmk2           DVWSVGCILAELLGSRPFFKGRDYVDQLNQILHVLGTPNEETLSRIGSPRAQEYVRNLPV 270
MgSlt2          DVWSVGCILAELLGSKPFFKGRDYVDQLNQILHYLGTPSESTLARIGSPRAQEYVRNLPY 270
AbSlt2          DVWSVGCILAELLGGKPFFKGRDYVDQLNQILHYLGTPNEETLSRIGSPRAQDYVRNLPY 270
Mpk1            DMWSVGCILAELLGGKPIFKGEDYVDQLNKILNLLGTPTEDTLRRVGSPRAQDYIRSLPI 277
                *:**.******:**.:*:*:*:*.***:***:**  **** :.** *:** ..*:*::.*
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```
SLT2            IPKVPFVNLYPNANSQALDLLEQMLAFDPQKRITVDEALEHPYLSIWHDPADEPVCSEKF 334
Mkc1            TRKASYEELFPDANPLALDLLERMLTLDPRERITVRDALNHKYLELWHDPKEEIECQVKF 355
Mgv1            MPKKPFPSLFPQANPDALDLLDKMLAFDPSSRISVEQALEHPYLQIWHDASDEPDCPTTF 330
Mps1            MAKKPFPTLFPNANPDALDLLDRMLAFDPSSRISVEQALEHPYLHIWHDASDEPDCPTTF 330
Cpmk2           MPKKNFATLFPQANPHALDLLDKMLAFDPSSRISVEQALEHPYLQVWHDPADEPNCPTIF 330
MgSlt2          MQKIPFHTLFRNANPDALDLLDRMLAFDPSQRIDVDEALEHRYLSIWHDASDEPNCPTTF 330
AbSlt2          MQKISFQSLFKNANPDALDLLDRMLAFDPSSRISVEEALEHRYLQIWHDASDEPSCPTTF 330
Mpk1            KPRVKFGTLYPNASPLALDLLSKLLTFDPAKRYGCEEALEHPYLAVWHDPADEPLCEVPF 337
                  :  :  *: :*.. *****.::*::** .*    :**:* ** :***. :*  *   *

SLT2            EFS-FESVNDMEDLKQMVIQEVQDFRLFVRQPLLEEQRQLQLQQQQQQQQQQQQQQQPS 393
Mkc1            DFKSFETVDGLDEMKQLIMDEVQKFREFVRKPIEEQQR-IQMQLHMQKREEQRQEEEEKE 414
Mgv1            NFD-FEVVEDVGQMRGMILDEVQRFRQNVRTVPGQSG-GGLQGQGVP------------- 375
Mps1            NFD-FEVVEDVGEMRKMILDEVYRFRQLVRTAPGAGGHGAPHAPQVP------------- 376
Cpmk2           NFD-FEVLDDVGEMRKVILDEVIRFRQMVRTASSAEPTTGQAQTAAAGQ------------ 378
MgSlt2          DFG-FEVVEEVPEMRQMILQEVRQFRQSVRAPQ-QQQYSQGHQQGQQ------------- 375
AbSlt2          DFQ-FEVVEEIPEMKKMILDEVSRFRQMVRV---QPGAGAGANQAPQ------------- 373
Mpk1            DFS-FEEEDSVSGMRDLILEEVRSFRYLVRQQTMPPVRKDSHELPPAPPAPQHPG----- 391
                :*  **  : :  :: ::::**  **  **

SLT2            DVDNGNAAASEENYPKQMATSNSVAPQQESFGIHSQN-LPRHDADFPPRPQESMMEMRPA 452
Mkc1            LLEQQRQFPAQESMDISQTPYNNLETNIGTPQVEDDYPRPQELDEFTFSNLESSSSMN-- 472
Mgv1            ------------------------VPLPQGNGQWTAEDPRPQEYAGHGNT------------ 401
Mps1            ------------------------IPAGAGQGQWKAEDPRPQEYVGQMN------------ 401
Cpmk2           ------------------------VPMPQAGGQWKAEDPRPQENTPQGN------------ 403
MgSlt2          ------------------------VPIPDGYDRT-HEDPRPQE---MGSTDPY-------- 400
AbSlt2          ------------------------VPIPNNYDRAGYEDPRPQEAFNQGGWNGS-------- 402
Mpk1            ------------------------AGVGPAFHEKRTAKAIWRNILGLHWRNSWRGRNYHR- 427
                                                          :.

SLT2            TGNTADIPPQNDNGTLLDLEKELEFGLDRKYF 484
Mkc1            --LFQDMAKPSGE-EYIKLEEELGFGLDWCYV 501
Mgv1            -----------------GLEQDLQGGLDASRR 416
Mps1            -----------------DLEAELAGGLDQRR- 415
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```
Cpmk2           -----------------GLEQDLQAGLDAA-- 416
MgSlt2          -----------------GLERELHDGLDAMR- 414
AbSlt2          -----------------DLERDLQGLDGRMR- 416
Mpk1            ---------------IIFVESVTVIGKHR--- 441
                                      :*
```

**C**

| | |
|---|---|
| Slt2 *(Saccharomyces cerevisiae)* | Identities = (67%), Positives = (80%) |
| Mpk1 *(Cryptococcus neoformans)* | Identities = (66%), Positives = (77%) |
| Mkc1 *(Candida albicans)* | Identities = (67%), Positives = (79%) |
| MgSlt2 *(Mycosphaerella graminicola)* | Identities = (86%), Positives = (90%) |
| Mgv1*(Fusarium graminearum)* | Identities = (83%), Positives = (87%) |
| Cpmk2 *(Claviceps purpurea)* | Identities = (80%), Positives = (85%), |
| Mps1 *(Magnaporthe grisea)* | Identities = (84%), Positives = (88%) |

**D**

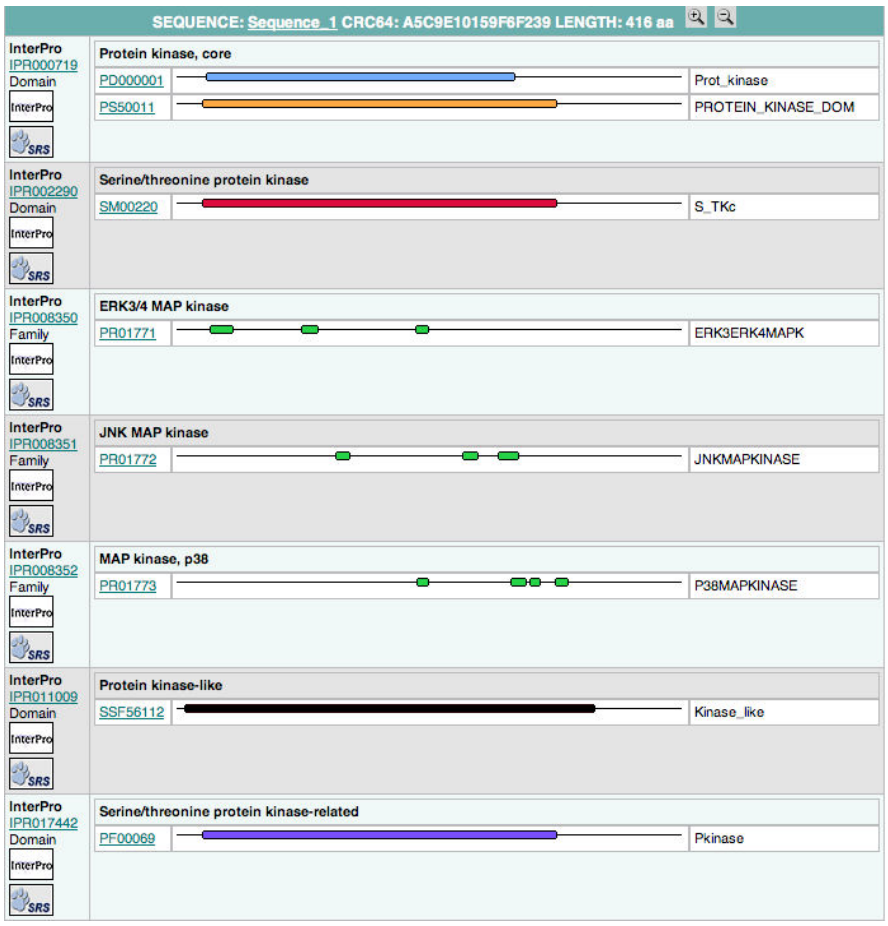


**FIG 4 A, Phylogenetic comparison of AbSlt2 MAPK homologs based on amino acid sequence alignment.** The known peptide sequence of *abSlt2* was compared with the MAP kinases Slt2, Mpk1, Mkc1, MgSlt2, *abSlt2*, Mgv1, Cpmk2, and Mps1. B, Alignment of the AbSlt2 amino acid sequence with the sequences of Slt2 homologs from other fungi. The conserved dual phosphorylation site TEY in all MAPKs is boxed and in red. C: Similarity of AbSlt2 to other Slt2 homologs. D. Results of Interpro scan depiciting conserved MAP kinase domains in AbSlt2.

**Targeted disruption of *abSlt2***

To elucidate the role of *abSlt2*, a knock out (KO) mutant was constructed by disrupting the target gene using linear gene disruption constructs (Cho *et al*., 2006).

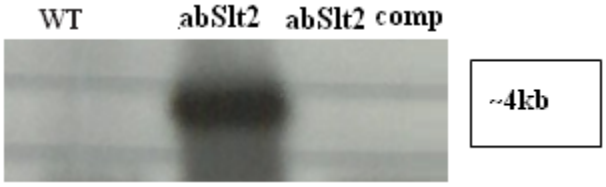


**FIG 5: Southern Blot of WT, abslt2, and abslt2 complement using Hyg B as probe**

PCR was used to verify successful transformation and integration into the target locus. The Slt2Comp primers were used because they begin amplification 678bp before the start codon of the Slt2 gene and 187bp after the stop codon. This design will yield a PCR product of 2.5kb with Alternaria WT template while the expected product size with the mutant template would be ~8kb. However, this lane should be blank on the gel because the taq polymerase will not amplify a target of that size very efficiently. The *abslt2* mutant was then complemented by re-inserting the gene into the genome. We confirmed no additional integration of the constructs in any other location in the genome by Southern hybridization with HygB.

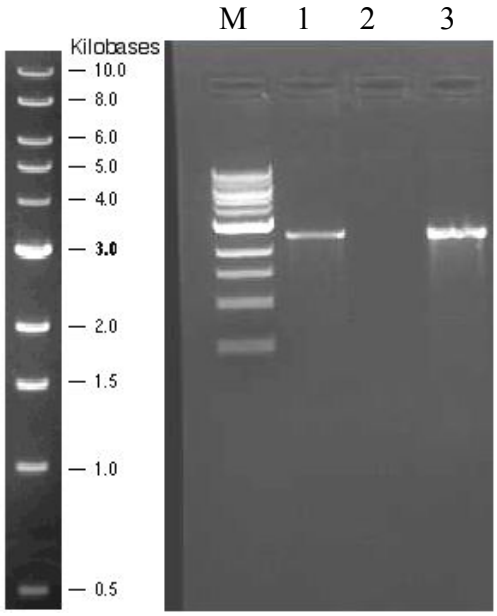


**FIG 6: PCR analysis confirms disruption of *AbSlt2*.** M=1kb marker, 1=WT DNA/Slt2comp primers, 2= *abslt2* DNA/Slt2comp primers, 3= *abslt2*comp DNA/Slt2comp primers. No amplification product was observed in Lane 2 indicating that transformant is disrupted at *AbSlt2* locus.

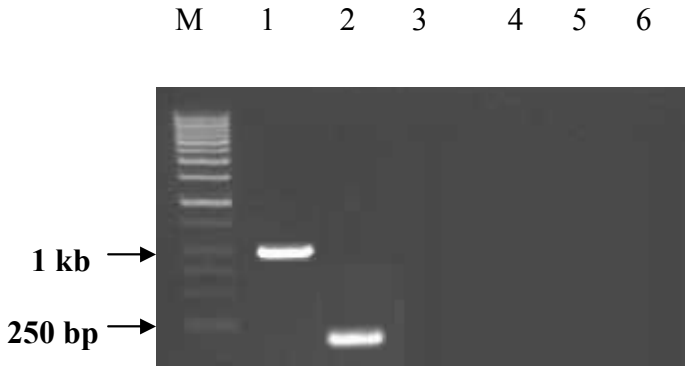


**FIG 7: PCR for Hygromycin B (*HygB*) gene amplification.** Lanes 1,2 depict amplification products obtained using *abslt2* genomic DNA as template and two independent primers sets for HygB gene amplification. Expected product size should be 1 kb (Hyg B.1 primer set) and 150 bp (HygB.2 primer set), respectively. Lanes 3,4 amplification products obtained using *abslt2* complement DNA as template using primer sets 1 and 2. Lanes 5,6 amplification products using wild type (WT) genomic DNA as

template with both primer sets. Note the absence of products when using WT genomic DNA and *abslt2* complement DNA as templates. M= MW marker.

***AbSlt2* is expressed in planta.**

The expression of *AbSlt2* was studied, with green cabbage DNA as the control, by reverse-transcription polymerase chain reaction (RT-PCR) during *in planta* conditions. cDNA specific primers were used resulting in an expected 166-bp amplification product.

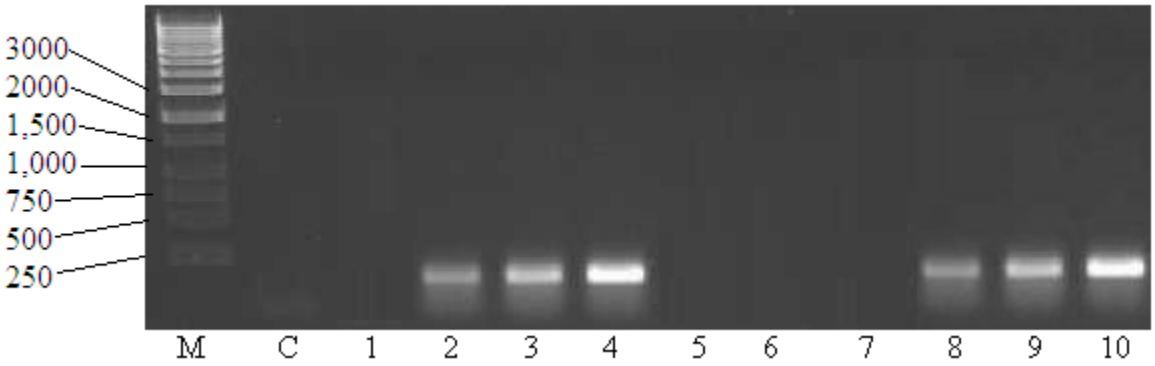


**FIG 8: RT-PCR of *AbSlt2* expression in infected Brassica plants. C, control (green cabbage genomic DNA). Lane 1, uninfected green cabbage cDNA. Lanes 2-4, green cabbage cDNA at 24, 48, and 72 hrs after *Alternaria brassicicola* wild type inoculation, respectively. Lanes 5-7 , green cabbage cDNA at 24, 48, and 72 hrs post *abSlt2* infection. Lanes 8-10, green cabbage cDNA at 24, 48, and 72 hrs post *abSlt2* complement infection.**

*In planta* expression analysis of *AbSlt2* reveals no expression of *AbSlt2* in the mutant strain confirming successful gene disruption. In contrast, expression of *AbSlt2* was clearly detected during infection of cabbage by wild type. Reverse-transcription polymerase chain reaction (RT-PCR) products were generated using gene-specific

primers for the *abSlt2* gene and green cabbage DNA as control. Lane M, 1-kb-plus ladder marker. Lane C, the control. Lane 1, first-strand cDNA generated from total RNA isolated from green cabbage. Lanes 2 to 4, first-strand cDNA generated from total RNA isolated from green cabbage inoculated with *Alternaria brassicicola* harvested at 24, 48, and 72 days post inoculation. Lane 5-7, first-strand cDNA generated from total RNA isolated from green cabbage inoculated with *abSlt2* harvested at 24, 48, and 72 days post inoculation. Lanes 8-10, first-strand cDNA generated from total RNA isolated from green cabbage inoculated with complemented *abslt2* harvested at 24, 48, and 72 days post inoculation. Ribosomal RNA bands stained with ethidium bromide (bottom panel) are shown before first strand cDNA synthesis.

**The effect of *abSlt2* on morphology, growth, and oxidative stress tolerance**

The *abslt2* mutant displayed different growth patterns when compared to the wild type (WT) on different media. After comparing fungal growth 7 days post inoculation (dpi), the mutant grew at a comparable rate on potato dextrose agar (PDA) compared to WT. On minimal media, the WT grew outward uniformly from the site of inoculation displaying well-spaced mycelia while the mutant displayed uneven growth and mycelia clumped together at the edges of growth.

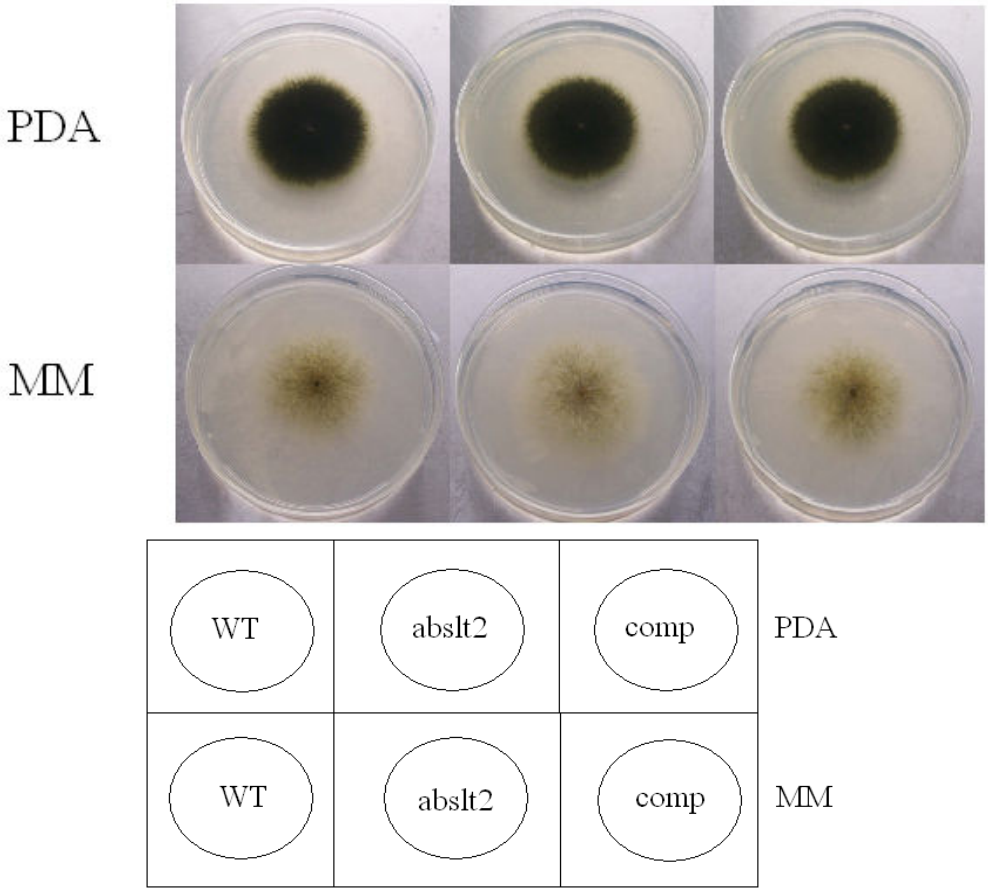


**FIG 9: Effect of *abSlt2* disruption on morphological characteristics of *Alternaria brassicicola*. Panels show the mutant and WT growing on PDA, and minimal media.**

To assess the mutant's ability to cope with oxidative stress, the same experiment was done using solid media PDA plates containing 2.5, 5, 10, 20, and 30 mM $H_2O_2$, respectively and compared 7 dpi. The mutant showed sensitivity to $H_2O_2$ at all concentrations while the WT displayed a slight increase in sensitivity at the 10mM concentration followed by increasing sensitivity at the 20 and 30 mM concentrations. Thus, disruption of *AbSlt2* results in increased sensitivity to oxidative stress.

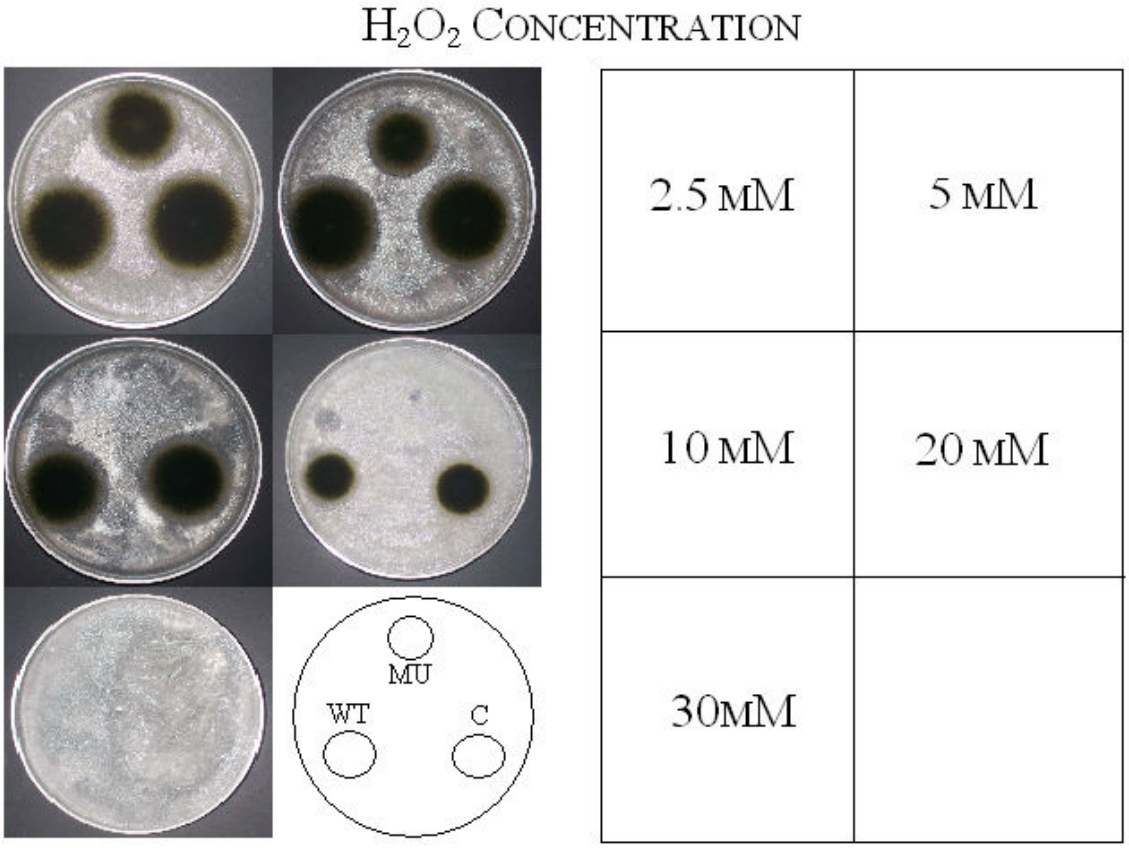


**FIG 10: Effect of *abSlt2* disruption on oxidative stress tolerance of *Alternaria brassicicola*. Panels show the mutant, WT, and complemented mutant growing on PDA with increasing concentrations of $H_2O_2$.**

### *abSlt2* mutants show decreased germination rate compared to WT

Germination rates were compared between WT, mutant, and complemented strains *A. brassicicola*. The fungi were cultured on PDA plates for 7 days then harvested into solutions of sterile water at a concentration of $1x10^4$ conidia/mL. 30 microliter drops were placed on a cover glass and allowed to incubate for 8 hrs at 25 degrees C. Germination rates were observed under microscope in three different experiments and

averaged. Wild type germination rate was 98%. The *abslt2* germination rate was 89% and *abSlt2* complement germination rate was 97%. Thus, germination rates appeared lower in the mutant compared to wild type and complemented strains but may not be statistically significant.

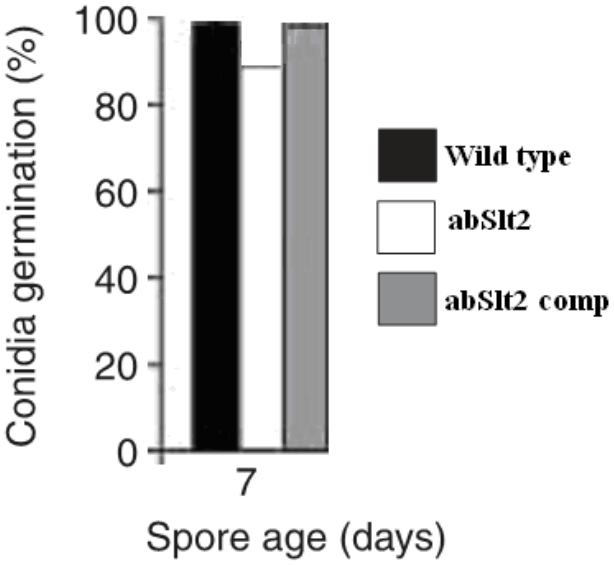


**FIG 11: Germination rate is slightly reduced in the *abslt2* mutant.** Germination test *in vitro*. Pathogenicity assay using conidia from 7-day cultures of wild type, *abslt2* mutant, and *abslt2* complement strains. The *abSlt2* mutants are severely reduced in virulence.

To determine if *AbSlt2* is essential to pathogenicity, a pathogenicity assay was conducted with the wild type (WT), disruption mutant and the complement by inoculation on the susceptible host plant *Brassica oleracea* (green cabbage). At 7 dpi, The WT and complement exhibited well developed lesions with a yellow halo surrounding the infection site. The mutant was not able to successfully invade the plant and did not advance outside of the initial infection site. Therefore, *AbSlt2* is critical for pathogenicity in *A. brassicicola* as has been observed for other plant pathogenic fungi.

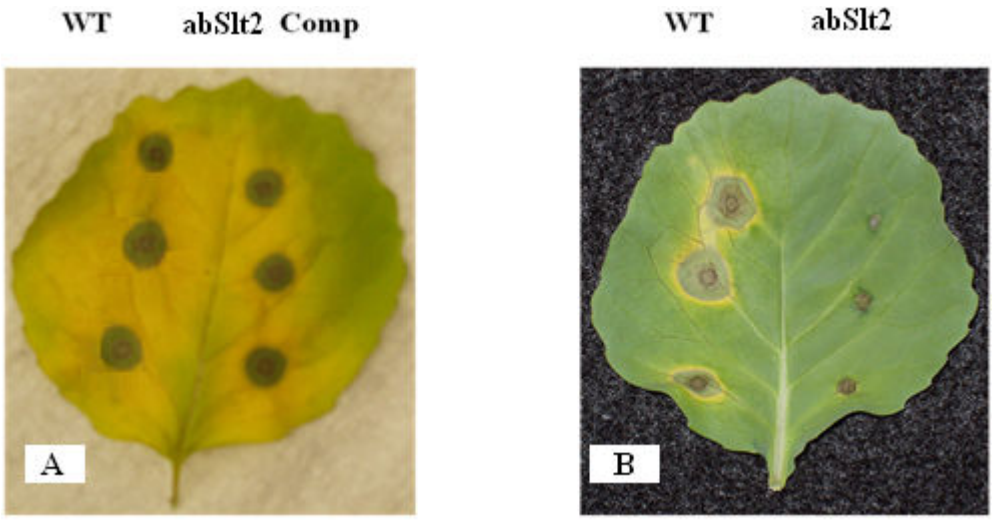


**FIG 12: Pathogenicity test of *abslt2* mutant.** A, 1 x $10^4$ conidia/mL of WT and *abslt2* complement at 7days post inoculation (dpi). B, 1 x $10^4$ conidia/mL of WT and *ablst2* at 7dpi.

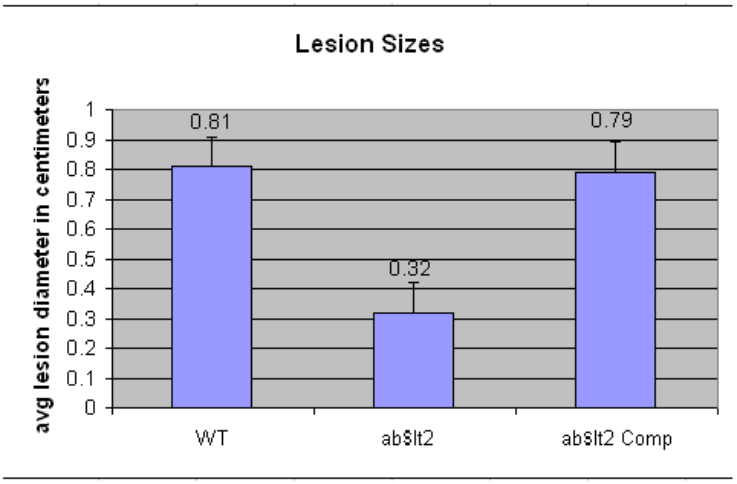


**FIG 13: Average lesion diameters of *abslt2* mutant compared to wild type and complemented mutant.** Size of lesions on green cabbage in centimeters after 7 dpi. Lesion size calculated by taking the average of 27 infection sites across numerous plants. Error bar depicts standard deviation.

**Inoculation on wounded site recovers pathogenecity in *abSlt2***

In order to determine if *abslt2* mutants could infect wounded plants, a sterile needle was used to slightly scratch the leaf surface of green cabbage prior to inoculations. The plant was then inoculated over that site with the *abslt2* mutant, *abslt2* comp, and wild type. Intriguingly, at 7 days post inoculation (dpi), all three fungal strains caused extensive disease symptoms, indicating that *AbSlt2* plays an essential part in fungal penetration of the plant.

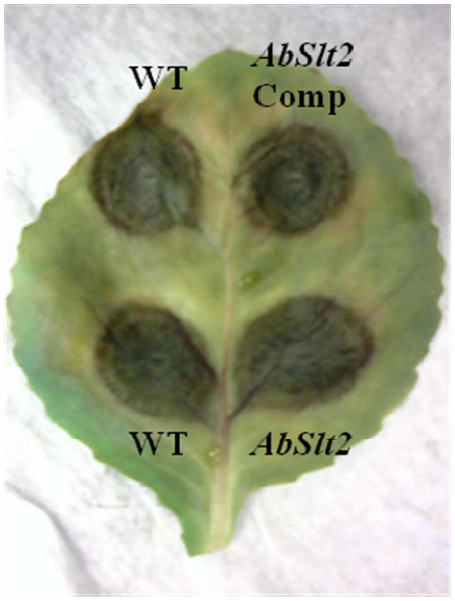


**FIG 14: Wounding restores pathogenicity of *abslt2*.** Pathogen Assay: 1 x $10^4$ conidia/mL of WT, *abslt2*, and *abslt2* complement (Comp) at 7days post inoculation on wounded sites.

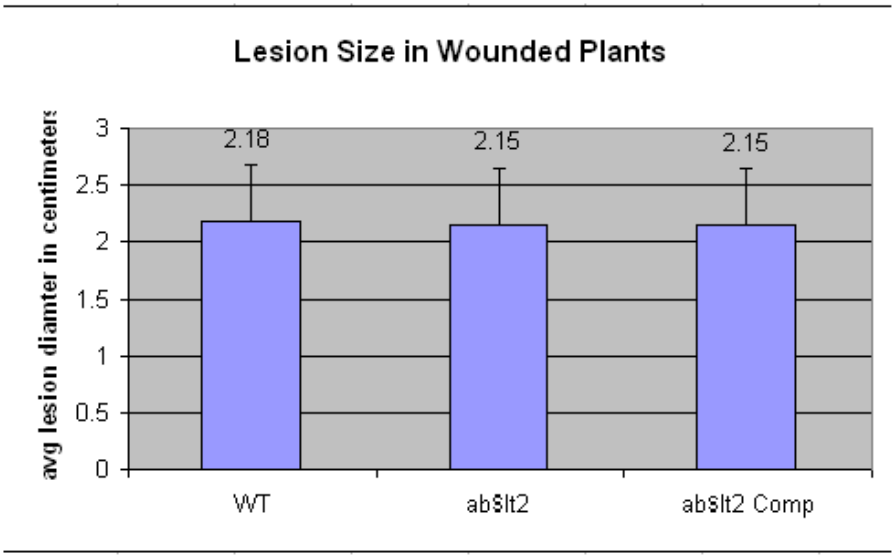


**FIG 15: Average lesion diameters on wounded plants inoculated with *abslt2* are similar to those caused by wild type.** Size of lesions on wounded green cabbage in centimeters 7 days posts inoculation. Lesion size calculated by taking the average of 27 infection sites across numerous plants. Error bar depicts standard deviation.

### *abslt2* disruptants display delayed filamentous growth, immature appresoria, and altered morphology

We inoculated green cabbage with 20 microliter drops of a $1x10^4$ conidia/mL suspensions of *abslt2* mutant and wild type (WT) and allowed them to grow for 24 and 48hrs. The samples were examined under a scanning electron microscope (SEM) at various magnifications. The mutant was impeded in its ability to grow across the surface of the leaf. This may explain why it does not produce a lesion outside of initial infection site. The mutant also displayed minimal appresorium formation. Appresoria that did form were slightly malformed and appeared to not be fully developed or immature. The mutant also appeared to seek out holes in the epidermis of the plant, such as stomates or natural wounds, to allow for easier penetration. This could explain why pathogenicity is

recovered in *abslt2* inoculated onto a wounded infection site. The basic structure of *abslt2* conidia are also altered when compared to WT conidia. The end of the conidium nearest the conidiophore is usually round while it tapers towards the apex. This gives the typical beak or club-like appearance of the conidia. The mutant conidia are more slender with no distinct tapering. Alternaria WT was able to fully colonize the leaf surface. It developed typical healthy, round appresoria that aids in fungal penetration of the leaf cuticle. It displayed quick, invasive filamentous growth while the mutant did not.

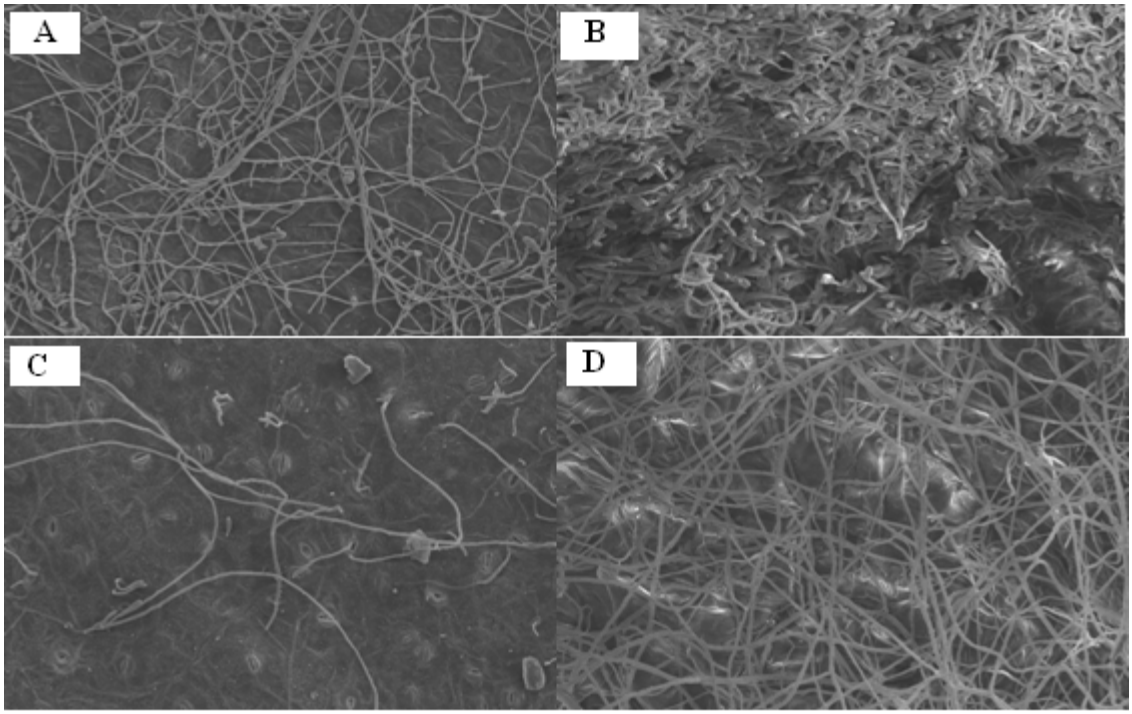


**FIG 16: Scanning electron microscopy (SEM) of Brassica leaf surface inoculated with wild type and mutant fungi.**

A, AB WT 24hrs pi, AB WT displays normal filamentous growth that encompassed the plant surface (600x mag). B, AB WT 48hrs pi, AB WT displays normal filamentous growth that complete engulfs the plant surface (600x mag). C, *abSlt2* 24hrs pi, *abSlt2* displays stunted hyhal growth. Does not have the same invasive capability of the WT.

(600xmag). D, *abSlt2* 48hrs pi, *AbSlt2* shows increased hyphal growth, but does not seem to be penetrating the leaf surface effectively.

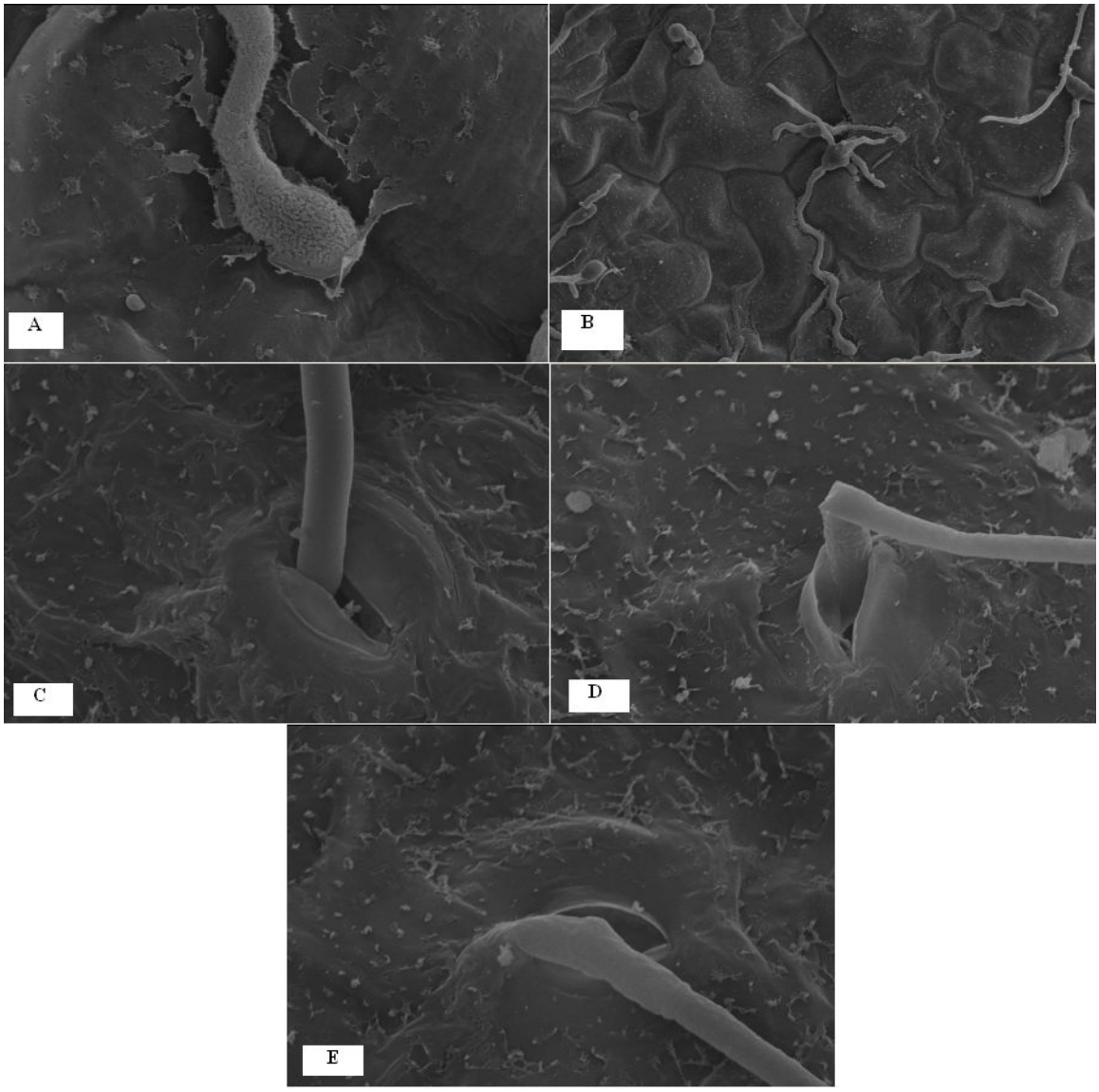


**FIG 17: Scanning electron microscopy (SEM) of host plant cuticle penetration events of wild type and mutant fungal appresoria.**

A, AB WT 24hrs pi, healthy appressorium penetrating the leaf surface (6.6x mag). B, AB WT 24hrs pi, hyphae spreading across the plant (8.27x mag). C, *abSlt2* 24hrs pi, malformed appressorium entering leaf through stoma (7x mag). D, *abSlt2* 24hrs pi, appressorium entering through opening on leaf surface (8.6x mag). E, *abSlt2* 24hrs pi, pointed, malformed appressorium (14x mag)

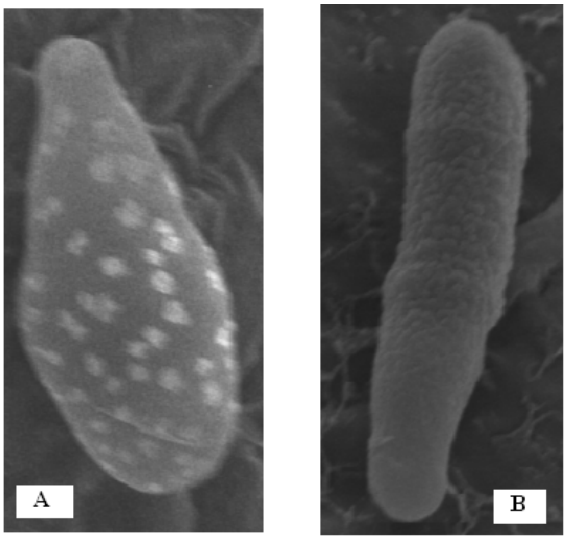


**FIG 18: 18. Scanning electron microscopy (SEM) of wild type and *abslt2* mutant conidia.**

A, Wild type, ungerminated conidia (5.6x mag). B, *abSlt2*, ungerminated conidia (8x magnification)

**DISCUSSION**

We have isolated and characterized *AbSlt2*, a homolog of the *Slt*2 MAP kinase that regulates cell wall integrity in *S. cerevisiae*. The observed expression pattern *in planta* suggests that *AbSlt2* is expressed during the initial stages of plant infection and

suggests that *AbSlt2* is vital to fungal pathogenicity. This study describes the role of *AbSlt2* in the life cycle and infection process of the important plant pathogen *Alternaria brassicicola.* Functional analysis of *AbSlt2* was performed using a combination of knockout mutagenesis (gene disruption) and phenotypic assays.

In advance of this study, the *Slt2* gene and corresponding protein has been characterized in many different phytopathogenic fungal organisms such as *Magnaporthe grisea*, *Colletotrichum lagenarium*, *Claviceps purpurea*, and *Fusarium graminearum*. However, these fungi represent a diverse group from across the fungal kingdom and represent various pathogenic lifestyles (Goodwin 2004; Goodwin et al. 2004). For example, the Slt2 homolog, MAF1, is required in *Colletotrichum lagenarium* for the early differentiation phase of the appresorium formation. Mutants failed to form appresoria (Kojima et al. 2002). However, the Slt2 homolog, mutants (*mps1*) of the rice blast fungus *Magnaporthe grisea* could form appresoria but were nonpathogenic because these appresoria were unable to penetrate the plant cell surfaces (Xu et al. 1998). *Claviceps purpurea* does not typically produce apressoria, but both *CMPK1* and *CMPK*2 (the Slt2 homolog) are necessary for penetration (Mey et al. 2002). In *Mycosphaerella graminicola, MgSlt2* was able to successfully penetrate through the stomata, but infectious hyphae were not able to branch out and colonize the mesophyll (Mehrabi et al. 2006). However, our studies showed that in *Alternaria brassicicola*, *AbSlt2* is required for normal appresorium development and hyphal colonization. These appresoria were able to penetrate the plant but at a reduced rate, yet preferred and actively sought to enter the plant through pre-existing openings in the leaf surface (stomata or wounds).

Once inside, the mutant did not establish invasive hyphal growth except on pre-wounded hosts. An explanation for this is that Slt2 functions to overcome plant defense after an elicitor provokes the system. These defenses include cell wall reinforcement, production of reactive oxygen species (ROS), and changes in extracelluar pH (Wojtaszek, 1997) at the site of infection. One function of the cell wall integrity kinase *AbSlt2* may be to protect the fungus against these types of plant defense responses by strengthening the fungal cell wall during invasive growth. The mutant was not able to penetrate the plant surface efficiently. This is why it appeared to actively seek alternative methods of entry (stomata and wounds). In *S. cerevisiae,* it has been demonstrated that alkaline stress rapidly and transiently activates Slt2 to regulate pH levels (Serrano et al. 2006). Without this gene, it is possible that *abSlt2* mutant cannot withstand the pH changes often occurring in the plant during defense responses. In addition, the *abslt2* mutant was also more sensitive to hydrogen peroxide than wild type and thus may be more sensitive to the host associated defense response, the oxidative burst. Mutants grew much slower than wild type at 2.5mM $H_20_2$ concentrations and ceased to grow at concentrations above 10mM while the wild type grew at concentrations as high as 20 mM. This may partially explain the lack of invasive hyphal growth *in planta* due to host derived ROS produced as part of the initial, rapid plant defense responses. Moreover, it took the mutant 48hrs to reach a similar saturation point of filamentous growth that was achieved in 24hrs by the WT thus giving the host additional time to mount an effective defense response prior to fungal penetration. In addition, by wounding the plant prior to inoculation, the mutant is able to bypass all the plant defense mechanisms at the surface and establish an infection directly beneath the plant surface.

Conidiation is significantly reduced in *M. grisea*, *C. lagenarium*, and *C. purpurea*, and at a later stage in *M. graminicola (*Kojima et al. 2002; Mehrabi et al. 2006; Mey et al. 2002; Xu et al. 1998). The *abslt2* mutant exhibits normal conidiation rates, even though the conidia display an aberrant shape. In *Emericella nidulans*, the *Slt2* homolog *MPKA* is involved in spore germination and polarized growth at several stages of colony formation (Bussink and Osmani 1999). Due to the slightly reduced rate of germination of *abslt2* compared to WT, we believe Slt2 participates to some degree in spore germination as well. In summary, these findings contribute to the understanding of pathogenicity mechanisms in the fungus *Alternaria brassicicola* and its role as a model system for plant pathogen interactions.

## MATERIALS AND METHODS

### Fungi.

*A. brassicicola* isolate ATCC96866, the isolate used for whole genome shotgun sequencing, was used in this study. *A. brassicicola* was cultured on 3.9% (w/v) potato dextrose agar (PDA) (Difco, Kansas City, MO) and 1% (w/v) glucose 0.5% (w/v) yeast extract broth (GYEB). Fungi were grown at 25 °C in the dark for both solid and liquid culture.

### DNA manipulation and analysis.

Basic DNA manipulations were according to standard protocols (Sambrook et al. 2001). Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Genomic DNA of *A. brassicicola* was prepared from freeze-dried spores using the DNeasy plant mini kit (Qiagen, Palo Alto, CA). DNA sequences were obtained from the unannotated *A. brassicicola* genome assembly, currently available for public ftp download (http:// genome.wustl.edu/). Homology searches were performed with the BLAST program (Altschul et al. 1997).

### Generation of targeted gene knock out mutants

Based on the full length cDNA sequence (AY705975) of *Slt2* homolog in *A. brassicicola* (*abSlt2*), two primers were designed at 308NT position (SltFHd, 5' gctaagcttatgaggagctcatggagtgc3') and 853NT position (SltRXb, 5'gcgtctagaggttggcgtttttgaatagc3') from the start codon with an exogenous enzyme site *Hin*dIII and *Xba*I, respectively. DNA sequences corresponding to genes used in this study

are available upon request from C. B. Lawrence. 564NT-long PCR products were amplified from genomic DNA and subsequently purified with PCR purification kit (Qiagen, Palo Alto, CA). Purified PCR products were digested with *Hin*dIII plus *Xba*I, and ligated at corresponding muticloning sites in the pCB1636 plasmid (Sweigard *et al.*, 1995) and pCB16G6 (Cho *et al.*, 2006). The plasmid constructs were transformed in *E. coli* strain DHα5 (Invitrogen, Carlsbad, CA). Plasmid was isolated from single transformant and used as template DNA to amplify linear gene disruption constructs using the pair of M13 forward and M13 reverse primers. It contained an antibiotic selectable marker gene Hygromycin cassette and the 546Nt long partial *Slt2* gene sequence.

**GFP-tagged construct and overexpression constructs.**

To make GFP-tagged LME disruption constructs, we added GFP cassettes in front of the hygB resistance cassettes carrying the 546Nt long partial target gene. Fungal transformation was carried out with linear PCR products based on the transformation protocol described previously (Cho et al., 2006).

***A. brassicicola* transformation.**

Transformation was carried out with either plasmid disruption constructs or linear PCR products based on the transformation protocol of *A. alternata* (Akamatsu et al. 1997), with modifications. Approximately $5 \times 10^6$ fungal conidia were harvested from a PDA culture plate and inoculated into 50 ml of GYEB (1% glucose and 0.5% yeast extract) media. They were cultured for 36 h with shaking at 100 rpm at 25ºC. The mycelia were harvested by centrifugation at 2,000 × *g* for 5 min and washed with 0.7 M NaCl followed by centrifugation again under the same conditions as before. The mycelia

were digested in 6 ml of Kitalase (Wako Chemicals, Richmond, VA, U.S.A.) at 10 mg/ml in 0.7 M NaCl for 3 to 4 h at 28ºC with constant shaking at 110 rpm. The protoplasts were collected by centrifugation at 700 × *g* for 10 min at 4ºC, washed twice with 10 ml of 0.7 M NaCl and then with 10 ml of STC buffer (1 M Sorbitol, 50 mM Tris-HCL, pH 8.0, and 50 mM CaCl2). The protoplasts were resuspended in STC at a concentration of 4 × 106 in 70 µl, after which 10 µg of plasmid or PCR products in 10 µl of ddH2O was added to the protoplast and gently mixed. The transformation mix was incubated on ice for 30 min. Heat shock transformation was performed by incubating the transformation mixture at 42ºC for 2 to 10 min. The transformation mix was incubated at room temperature after the addition of 800 µl of 40% PEG solution. Then, 200 µl of the transformation mixture was added to 25 ml of molten regeneration medium (1 M sucrose, 0.5% yeast extract, 0.5% casein amino acids, and 1% agar,) in a 50-ml tube and subsequently poured into a 100-by-15-mm petri dish. After 24 h, the plates were overlaid with 25 ml of hygB (Sigma-Aldrich, St. Louis, U.S.A.) containing PDA at 30 µg/ml. Individual hygB resistant transformants were transferred to a fresh hygB-containing plate between 10 and 15 days after each transformation. Each transformant was purified further by transferring a single spore to a fresh hygB-containing plate. The mutant was then complemented by reintroducing an intact Slt2 gene into the mutants genome by the same process of transformation. Slt2Comp F&R primers were used.

**DNA isolation**

*A. brassicicola* was cultured for 2–3 days in 50 mL GYEB media. Approximately 0.2 g mycelia was harvested and filtered with Miracloth (Calbiochem, Darmstadt, Germany), semi-dried with paper towels, and ground into fine powder with a mortar and

pestle in the presence of liquid nitrogen. Total genomic DNA was extracted using the Plant DNeasy kit (Qiagen, Palo Alto, CA).

**Southern hybridization**

A total 2–3 μg of genomic DNA was digested with an endonuclease BsrGI (New England BioLab, Beverly, MA). The digested DNA was size-fractionated on a 0.7% agarose gel, followed by overnight transfer to a Hybond N+ nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The transferred DNA was UV cross-linked at 120 mJ (Spectronics Corp., Westbury, NY) to the membrane and subsequently hybridized with 0.5-kb-long probes that were amplified from *A. brassicicola* genomic DNA using PCR DIG Probe Synthesis Kit (Roche Diagnostics, Mannheim, Germany). The entire procedure from the hybridization to the signal detection was carried out with Block and Wash Buffer Set and DP-Star in the DIG Detection Kit (Roche Diagnostics) according to the manufacturer's protocols with following specific details. Hybridization was performed at 50 °C. After the hybridization, the membrane was briefly rinsed three times at room temperature in wash solution 1 (1× SSC, 0.1% SDS) and stringently washed at 68 °C in wash solution 2 (0.1× SSC, 0.1% SDS) for 30 min.

**Expression analysis**

Expression analysis of the *AbSlt2* gene was performed in planta at different time points using RT-PCR. Samples were collected from inoculated green cabbage plants that were subsequently flash frozen and ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted from 20 mg of ground leaves using the RNeasy plant mini kit (Qiagen, Palo Alto, CA) according to the manufacturer's instructions followed by DNase digestion for 15 min at 37 °C using DNase Mini Kit (Promega, Madison, WI). First-

strand cDNA was conducted using Invitrogen Super Script Reverse Transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. PCR reactions were subsequently carried out with 2-µl aliquots of this reaction in a 25-µl PCR reaction volume, using cDNA specific primers. Based on the full length cDNA sequence of the *Slt2* homolog in *A. brassicicola*, two primers were designed at 795 NT position (Slt2RTPCRFwd, 5'tcgcaacctaccgtacatgcagaa3') and 961NT position (SltRTPCRRev,5'aggcatcgtgccagatttgtaggt3') from the start codon. The samples were visualized on 0.7% agarose gel using 10-µl aliquots of the PCR products.

**Germination rate comparisons**

Conidial germination was measured on cover glasses (Fishier Scientific, Hampton, NH). Conidia were harvested from 7 day-old cultures on PDA in sterile distilled water and adjusted to $1 \times 10^3$ conidia/ml. 30 µl drops were placed on cover glasses, and then incubated at RT for 8 hrs on cover glasses. The slides were then examined under the microscope. The percentage of germinated conidia was determined by examination of at least 100 conidia in at least three independent experiments.

**Microscopy**

$1x10^4$ mL conidia of 7 day old wild-type Alternaria and *abSlt2* were inoculated on green cabbage and allowed to propagate for 24 and 48 hrs. The infection site was excised with a scalpel and placed in a fixative composed of 5% glutaraldehyde and 3% formaldehyde in 0.1m Na Cacodylate buffer with 2.75% picric acid overnight at 4 °C. After washing three times with 0.05 M sodium cacodylate buffer (pH 7.2) for 10 min each, samples were post-fixed with 1% (w/v) osmium tetraoxide in the same buffer for 2 h at 4 °C. The samples were then dehydrated in a graded ethanol series and critical point dried.

Because the samples were poor conductors of electricity, they were mounted and coated with gold in order to eliminate image distortion due to charging. The samples were visualized at different magnifications using a ZEISS EVO 40 (Carl Zeiss AG, Oberkochen, Germany) scanning electron microscope operating at 12 kV.

**Growth Assays**

$1x10^4$ spores/ml of *abSlt2* and *A. brassicicola* Wt were inoculated on different solid media plates in 10 µl drops. The growth rates were compared after 7 days post inoculation (dpi) on PDA, Minimal Media, and Water Agar plates. The same amount of spores were also grown on solid media plates containing 2.5, 5,10, 20, 30 mM $H_2O_2$, respectively. Growth rates were compared 7 dpi.

**Pathogen Assays**

PDA plates of *abSlt2, abSlt2* Complement, and *Alternaria brassicicola* WT were inoculated and allowed to incube for 7 days at 25 degrees Celsius. After 7 days, the plates were harvested and $1x10^4$ spores/mL of *abSlt2, abSlt2* Complement, and WT were inoculated onto 6 week old *Brassica oleracea* (green cabbage). The plants were stored at room temperature in a moist container for 7 days.

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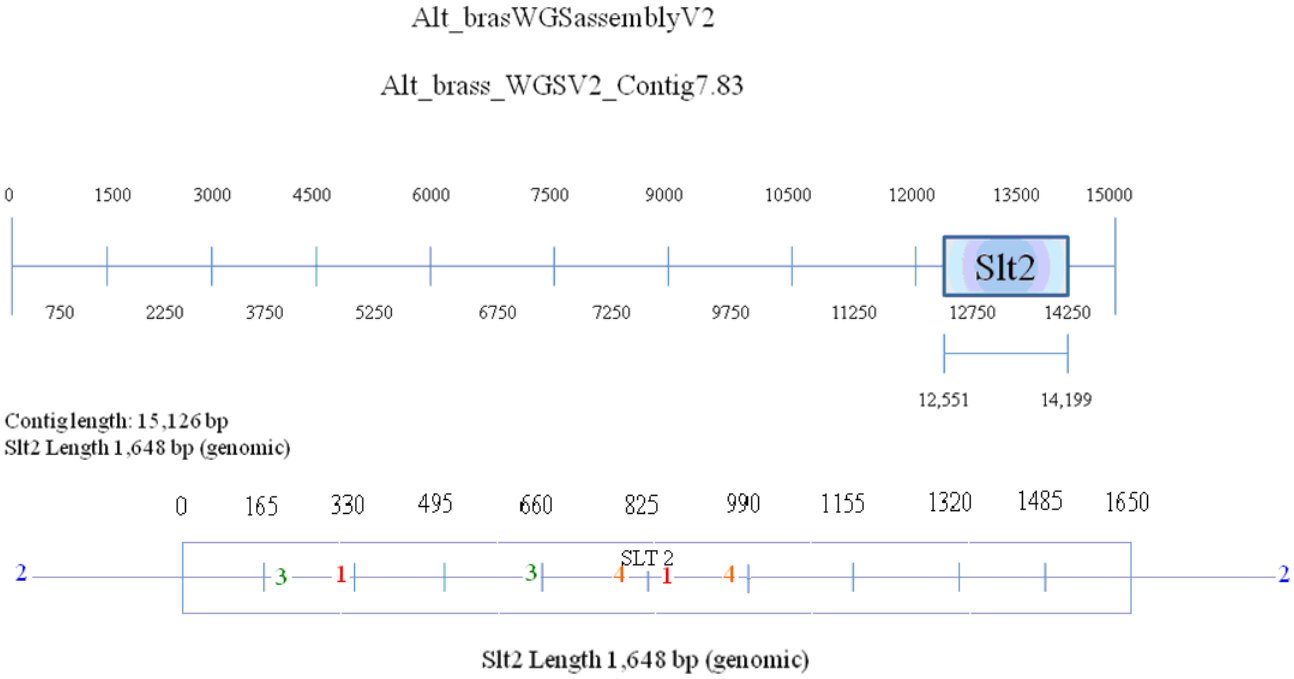
## APPENDIX

## PRIMERS USED IN THIS STUDY AND GENOMIC INFORMATION




**1. Slt2 KO Primers**
**2. Slt2 Complement Primers**
**3. Slt2 Comp. Confirmation Primers**
**4. Slt2 RT-PCR Primers**

**Slt2 KO Primers**
These primers were used to amplify a fragment of the Slt2 gene to be used in fungal transformation. Hind III and Xba I were added to the beginning of the primers to aid in restriction digests.

LEFT PRIMER ATGAGGAGCTCATGGAGTGC
**Slt2FHd GCT AAGCTT ATGAGGAGCTCATGGAGTGC**
RIGHT PRIMER GGTTGGCGTTTTTGAATAGC
**Slt2RXb GCG TCTAGA GGTTGGCGTTTTTGAATAGC**
SEQUENCE SIZE: 1251
INCLUDED REGION SIZE: 1251

PRODUCT SIZE: 546, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 2.00
EXCLUDED REGIONS (start, len)*: 1,300 900,350

```
1 atgggcgacctcgccaaccgtaaagtgttcaaggtgttcaaccaagagttcatcgtcgac
```

```
    XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
 61 gagcgctacaatgtgaccaaggagctgggacagggtgcctatggcatcgtttgtgcggct
    XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
121 accaacaaccagacaggcgaaggcgtcgccatcaagaaggtcaccaacgtcttcagcaag
    XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
181 aagatccttgcgaagcgcgccctgcgcgagatcaagctgctccagcacttccgaggccac
    XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
241 cgcaacattacatgtctctacgacatggacattccccgcccggacaacttcaacgagtgc
    XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
301 tacctctatgaggagctcatggagtgcgacctggcagccatcatccgctctggccagccg
           >>>>>>>>>>>>>>>>>>>>
361 cttaccgatgctcacttccagtccttcatctaccagattctgtgcggcttgaagtacatc
421 cattccgcaaacgtccttcatcgcgatctgaaacctggcaacctcctcgtcaacgccgac
481 tgcgagctcaagatttgcgacttcggtctcgcaagaggattctccatggatcctgaggag
541 aacgcaggttacatgactgaatatgtcgctacccgatggtacagagctcctgagattatg
601 ttgagtttccagagctacacgaaagctatcgatgtatggtcagtaggctgcatcctcgct
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                                                        <<<<<<<
841 aaaaacgccaaccggacgcgctcgatttgctcgaccgcatgctcgctttcgacccgtca
    <<<<<<<<<<<<<                                           X
901 agccgtatttcagtcgaggaagctctagagcacaggtacctacaaatctggcacgatgcc
    XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
961 tccgacgagccttcctgcccaacgacattcgacttccaatttgaggttgttgaggagatt
    XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
1021 ccagagatgaagaagatgattctagacgaggtcagccgcttccgccagatggttcgtgta
     XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
1081 caacccggtgcaggcgctggggcgaaccaggcgccgcaagtcccaatacccaacaactac
```

```
      XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

1141 gaccgtgccggctatgaagacccaaggccacaagaagcgttcaaccagggtggatggaac

      XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

1201 ggcagcgacttggagcgtgaccttcagggccttgacggtcgtatgcgatga

      XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
```

**Slt2 Complement and Confirmation Primers**

These primers were used to amplify up and downstream of the start and stop promoters of the Slt2 gene. This would ensure that the entire Slt2 gene would be reinserted into the genome after complementation of the *abSlt2* mutant. The confirmation primers were used to confirm successful reintegration of the Slt2 gene.

SLT2 genomic DNA sequence

SLTcompl_For ATGCTGTAGATGCCCTCGTT
SLTcompl_Rev CGAAGACCTCTAGGCGACAC
PRODUCT SIZE: 2514

SLT2 comp conf primers
3. 5'GCGACGCATACGAATACTGA3'
3. 5'CTACGCAAAGTCAGCCATGA3'
PRODUCT SIZE: 841

Genomic DNA sequences including promoter and terminator

```
TCCAACTGCCGCCCTATCGTGCTCGCGATGGAAGACGCACCAAATCTGATGACGATGCCCTTTCCCCCGTC
TCGCCTTTGGAGGGGCCTTTGGAGGGGTCTACAGATACCTCCCGACAGCGATTCTCCTACGAGTCATGAAT
CCCCCAAAGCTAGTTGTTTAGCGACATTACGACTGGCGTTTACTTTGTTTGGCATAACTGGCGGGGTTCTC
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TGCACGACAAGCTGCGCGCTGCGCTAGGCTCATGCGTTCATCTTGATTGACATGCACGCACGCACGCACGC
ACTGAGGAACCGGTCTGGCCGTCGGCTGGCTAAAGGGTCATGGGCCTGCAACAGGCAGTTGTCCTGGTTGT
AGGGCAGAGCCGGCGGCGCGGGTGAAGCGGTCTGAGGAAGGTGGCTATAGCAGCGCCTTGCCGCCTTGGGC
CAAGTCGAACCCCTCGTATGCGCTGCGACTGGATACCAACCCCACCATAACCCTCACGGAGGCCGCGACGA
GGCACAGGACGACCGCTTTTCTGCATCGTCGCTTTGTCAATCGCTTCCCCATTGCTGCGTACTTCCCGGTG
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GATGCTAAACGCCGTTGCACAGATTACATGTCTCTACGACATGGACATTCCCCGCCCGGACAACTTCAACG
```

AGTGCTACCTCTATGAGGGTATGGATTCTCCCTCTCGATATTCAATCAACAAGGAGACACTGACATGGTGC
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CCTTCATCTACCAGATTCTGTGCGGCTTGAAGTACATCCATTCCGCAAACGTCCTTCATCGCGATCTGAAA
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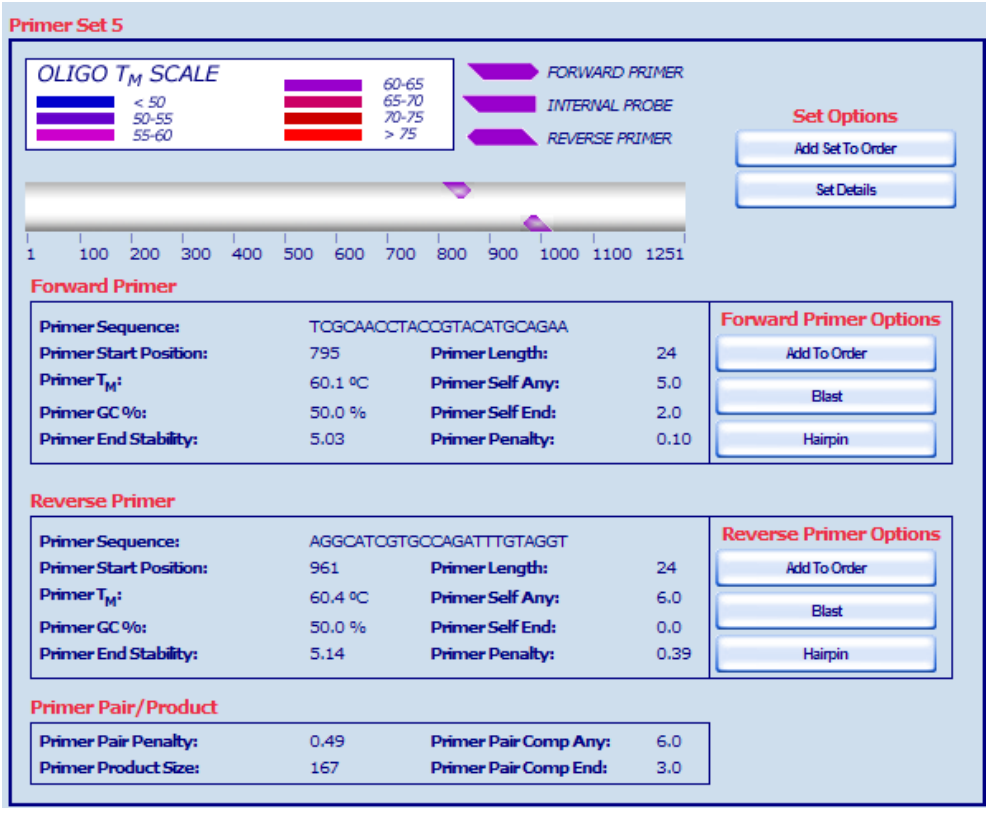
## Slt2 RT-PCR Primers

These primers were designed specifically to amplify a small region of Slt2 cDNA during RT-PCR



**Slt2 Hyg Primers**
These primers were used to confirm successful mutant transformation by amplifying the hygromycin B phosphotransferase gene that is only present in mutant strains.

LEFT PRIMER GATGTAGGAGGGCGTGGATA
RIGHT PRIMER ATAGGTCAGGCTCTCGCTGA
SEQUENCE SIZE: 1026
PRODUCT SIZE: 150

**Full length genomic sequence of contig with Slt2 identification (including PstI restriction sites)**

PstI sites in Contig 7.83

| # | MS | Cut position | Site with flanks |
|---|---|---|---|
| 1 | | 4075/4071 | 4061 cgtctagaac C_TGCA^G tgcgaagggg |
| 2 | | 5311/5307 | 5297 atagaaccac C_TGCA^G tgagcaatat |
| 3 | | 6559/6555 | 6545 gttccgtctc C_TGCA^G tgataggaga |
| 4 | | 7303/7299 | 7289 ggtgagtagg C_TGCA^G atcgataggc |
| 5 | | 10722/10718 | 10708 caggaccttt C_TGCA^G gaggcctatt |
| 6 | | 14954/14950 | 14940 atgtcgcccg C_TGCA^G tcgatccagg |

>lcl|Alt_brass_WGSV2_Contig7.83

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CGCGCAGACGCGCGCAAGGAAAAGAAGCCGTCGTAGCCGAGCCTGTTGTT
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