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IDENTIFICATION AND CHARACTERIZATION OF SUPPRESSORS OF
HOPM1; A PLANT PATHOGEN EFFECTOR OF *PSEUDOMONAS*
SYRINGAE PV. *TOMATO* DC3000

by

Vanessa Revindran

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Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
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Advisor: John R. Geiser, Ph.D.

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Vanessa Revindran

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HOPM1; A PLANT PATHOGEN EFFECTOR OF *PSEUDOMONAS*
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Vanessa Revindran, Ph.D.

Western Michigan University, 2012

We have created a yeast model system to study the action of the plant pathogen effector HopM1 in *Saccharomyces cerevisiae*. *Pseudomonas syringae*, causative agent of bacterial speck in tomatoes, utilizes the type III secretion system to shuttle the effector proteins into the host cell.

When expressed in yeast, HopM1 is lethal on solid media at 21°C, but not at 30°C and 37°C. The same temperature sensitive ability of HopM1 to cause death on solid media is also observed in liquid. As demonstrated by SDS PAGE-Western blot analysis, HopM1 protein is present at 21°C, 30°C and 37°C. At 21°C, a full-length protein of 78kDA is observed. At 30°C and 37°C, the majority of HopM1 protein exists as degraded fragments. HopM1 containing strains were visualized using the V5 epitope and immunofluorescent microscopy. HopM1 localizes to mitochondria and secretory organelles. This result was confirmed using cellular fractionation and sucrose

gradient density centrifugation. When plated on media containing glycerol, we observed no change in expression of HopM1, thus indicating that it is unlikely that binding to mitochondria results in the lethal phenotype.

We have isolated 19 spontaneous suppressor strains that are capable of surviving the HopM1 imposed lethality at 21°C. All strains have been examined for HopM1 protein expression, of which 13 express full-length HopM1 at 21°C, and 5 do not. SupM1-16, showed a significant increase in growth rates as compared to the wild type strain expressing HopM1. None of the suppressor strains show a change in localization of HopM1 as compared to wild type. One of the suppressor strains, SupM1-16 was sequenced to identify the gene(s) responsible for the suppression phenotype. Six genes that may be the suppressor gene were identified. The most likely candidate is *RSP5*; an E3 Ubiquitin Ligase. *RSP5* contains a single mutation that changes a Glycine to Valine in the HECT domain. Overall our findings suggest that HopM1 kills the yeast cell by perturbing a secretory pathway regulator and that mutation of *RSP5* alters HopM1 effects on this pathway to allow survival.

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CHAPTER 1

INTRODUCTION

All around the globe, people go hungry every day. In 2009, it was estimated that more than 1 billion people went without food (Food and Agriculture Organization of the United Nations, 2009). The United Nations estimates that about 850 million people go to bed hungry each night, and of that, 50 million are in the United States (Fighting Hunger Worldwide, 2010, World Hunger Education Services, 2010).

There are many causes as to why many people are left without food. Among them; having being hit by natural disasters such as earthquakes and storms, violence and war and the fact that there is just not enough food to accommodate the demand. Besides that, the persistently high price of food and low production due to crop loss are also important reasons as to why people go hungry every day (Fighting Hunger Worldwide, 2010, Rosegrant, 2008, World Hunger Education Services, 2010).

The increasing use of crops for non-food purposes has become a contributing factor to the decrease in the amount of food available. The usage of food crop for biofuel

production has taken its toll on the amount available for consumption. The reason biofuels have gained a lot of attention stems from the fact that biofuels can be harvested from crops easily grown by farmers (Doornbosch and Steenblik, 2008). On top of that, the sheer increase in demand for and production of biofuels has had a significant impact on the supply and demand for food crop as biofuels (Rosegrant, 2008).

Another huge factor that contributes to the decrease of food crops available for consumption is due to plant disease. Worldwide crop loss due to plant disease has been estimated as 36.5% of production capacity (Agrios, 2005). These losses are much higher in developing countries and much lower in developed countries. It has been estimated that of the 36.5% of the average crop loss, 14.1% is caused by pathogens, 10.2% by insects and 12.2% by weeds. These numbers put the total of annual worldwide crop loss from plant disease at \$220 billion (Agrios, 2005). In the United States alone, it has been estimated that crop loss due to disease is worth \$9.1 billion (Agrios, 2005).

Finding drugs or compounds capable of reducing pathogen effect and even preventing it, would help increase food crop production. This is due to the fact that with the advent of biotechnology and with the discovery of biofuels,

farmers have slowly begun to increase the price of crops (Koh and Ghazoul, 2008, Pimentel et al., 2009). Many farmers are solely looking at producing crop for biofuel purposes, thus taking away from the total amount of food crop available for consumption (Koh and Ghazoul, 2008).

Plant Diseases

Plant diseases are caused by many factors, among them abiotic and biotic stress. Abiotic stressors are caused by non-living factors, such as drought stress, sunscald, freeze and wind injury, chemical drift, nutrient deficiency and even improper practices such as overwatering and planting too deep (Grant et al., 2006, Mittler, 2006). Living organisms such as fungi, bacteria, viruses, nematodes, insects, mites and animals are the main culprits behind biotic stress. (Grant et al., 2006, Mudgett, 2005). Insects cause serious damage by chewing, and by doing so, induce a wound response that includes the production of protease inhibitors and alkaloids (Dangl and Jones, 2001). Nematodes on the other hand have a more refined method of parasitism. They do so by administering a developmental response on plant cells that inadvertently lead to the growth of galls, cysts or root knots (Dangl and Jones, 2001).

Three critical factors need to be present in order for plant disease to occur, a susceptible host plant, a biotic stressor and the ideal environmental conditions. The interaction that occurs between these 3 components is known as the "disease triangle" (Agrios, 2005). If a virulent pathogen is introduced into a susceptible host lacking optimal environmental conditions, disease development may be prevented (Parker and Gilbert, 2004). There are many routes of entry for a pathogen into its host. For example, viruses invade a plant cell intracellularly and mycelium are produced and grow through the host cell in the case of fungi. (Buttner and Bonas, 2002 and Galan and Collmer, 1999).

Plant Basal Defense

Plants have evolved numerous ways to respond to exterior attacks. Among them are preformed barriers such as thicker waxy cuticles, trichomes (specialized epidermal cells that are present in most plants and usually appear as fine hairs on the exterior), presence of secondary metabolites (chemical compounds that are very distinct from the intermediates and the products of primary metabolism), and an inducible basal defense response that is able to suppress pathogen/microbe growth (Alfano and Collmer, 1997,

Bennett and Wallsgrove, 1994, Levin, 1973). Plants have also evolved the ability that allows them to act as suppressors of programmed cell death, suppressors of plant cell wall remodeling, and as activators of the Jasmonic Acid pathway (Alfano and Collmer, 1997, Greenberg and Vinatzer, 2003, Loake, 2001, Mudgett, 2005).

Programmed Cell Death (PCD)

Programmed Cell Death (PCD), is a physiological process of cell death that is performed by the plant. It involves the destruction of cells that are damaged and not functioning correctly (Greenberg, 1996, Greenberg, 1997, Pennell and Lamb, 1997). This process is necessary for growth and survival in plants, and occurs locally and sometimes even on a wider scale (Pennell and Lamb, 1997). Plants are capable of recognizing certain pathogens and upon recognition defenses that result in the limitation of pathogen growth are activated (Greenberg, 1996, Greenberg, 1997). Programmed cell death is therefore an essential process in safeguarding proper plant development and in ensuring that appropriate defense responses are elicited against pathogens (Greenberg, 1996, Greenberg, 1997).

Recent studies have shown that both AvrPto and AvrPtoB (both effector proteins of *Pseudomonas syringae*) interrupt

the Pto disease resistance pathways by acting as a suppressor of PCD (Abramovitch and Martin 2005, Abramovitch et al., 2003, Jamir et al., 2003, Loake, 2001, Mudgett 2005). AvrPto and AvrPtoB interrupt the Pto disease pathway by interacting with the Pto serine/threonine kinase, a resistance protein in tomatoes. Once AvrPto and AvrPtoB are recognized in the tomato host, the Prf-dependent signal transduction pathway is activated and this leads to disease resistance by the elicitation of the Hypersensitive Response (Abramovitch et al., 2003, Mudgett, 2005, Pedley and Martin, 2003). In *N. benthamiana* however, the expression of AvrPtoB and AvrPto fails to elicit the Hypersensitive Response. The Hypersensitive Response (HR) is defined as cell death that is localized at the site of infection during an incompatible interaction between a resistant plant and an avirulent pathogen (Dangl and Jones, 2001, He, 1996). These results therefore indicate that AvrPtoB may act as a specific suppressor of the Pto pathway in *N. benthamiana* but not in tomatoes (Mudgett, 2005, Pedley and Martin, 2003).

Plant Cell Wall Remodeling

The plant cell wall is the first layer of the defense that is encountered by a pathogen when trying to gain

access into a plant (Maor and Shirasu, 2005). Upon attack, the plant reinforces its cell wall in order to slow down or even prevent pathogen attack. Reinforcement of the cell wall is done via callose deposition (callose is a sugar polymer that consists of 1-3- β -D-glucan subunits) at the site of attack (Maor and Shirasu, 2005). Another way in which plants defend themselves against pathogen attack is by remodeling and repairing wounds to their cell walls by forming thick protrusions known as papillae or even by the production of wound plugs (Alfano and Collmer, 1996, Bent, 1996, Hauck et al., 2003).

The effector protein AvrPto blocks the induction of papillae formation via the Salicylic acid independent pathway (Grant et al., 2006, Hauck et al., 2003, Mudgett, 2005). Besides AvrPto, HopM1 and AvrE are also the other known effectors of *Pseudomonas syringae* pv. *tomato* that are capable of suppressing the papillae formation by preventing the deposition of callose at the site of wounding/infection (Alfano and Collmer, 1996, Lindgren, 1997, Mudgett, 2005, Nomura et al., 2006).

Salicylic Acid and Jasmonic Acid Pathway

Salicylic Acid plays an important role as the plant signaling molecule that is responsible for both local and

systemic induced disease resistance (Durner et al., 1997, Mudgett, 2005). The local induced resistance is expressed as a hypersensitive response through the growth of lesions that prevent the pathogen from spreading (Pieterse and van Loon, 1999). Jasmonic Acid on the other hand is a signaling hormone that is induced and immediately produced as a response to herbivores and wounding (Clarke et al., 2000, Pieterse and van Loon, 1999, Wasternack et al., 2006, Zhao et al., 2003,). The activation of JA signaling suppresses the SA signaling pathway, thus making plants more susceptible to pathogen attack (Mudgett, M.B., 2005).

Salicylic Acid signaling is required for effective defense against pathogen infection in *Pseudomonas syringae* (Clarke et al., 2000, Grant et al., 2006, Mudgett, 2005). The phytotoxin Coronatine (COR), which is found in *Pseudomonas syringae*, acts as a mimic of JA, and suppresses the SA signaling pathways, thus preventing the pathogen from being killed (Brooks et al., 2005, Duner et al., 1997, Mudgett, 2005).

Resistance Protein Activation

Resistance proteins or sometimes referred to as R proteins, play a key role in the defense signal transduction pathways (Mudgett, 2005). These resistance

proteins recognize a matching pathogen avirulence protein and confer upon the plant the specific innate immunity (Takken et al., 2006, 2009). There are numerous classes of R proteins, among which are Pto-Serine/Threonine Kinases, Leucine Rich Repeat-Nucleotide Binding Sequence-Leucine Zipper motifs and Extracellular Leucine Rich Repeats (Bent and Mackey, 2007, Takken et al., 2006, 2009).

Many pathogens have however evolved numerous ways to suppress the host basal defense responses (Takken et al., 2006, 2009). One well-studied example is the suppression of the RIN4 regulated basal defense by *Pseudomonas syringae* in *Arabidopsis* (Lim et al., 2004, Mudgett, M. B., 2005, Takken et al., 2006). The effector AvrRpt2 of *Pseudomonas syringae* pv. *tomato* plays a role in suppressing the activation of the RPM1-dependent disease resistance pathway in *Arabidopsis* (Bent, 1996, Bretz and Hutcheson, 2004, Chen et al., 2000, Lim and Kunkel, 2004, Takken et al., 2000, 2006, 2009). The RPM1-dependent disease resistance pathway functions by recognizing the presence of the effector protein AvrRpt2 (Maor and Shirasu, 2005, Mudgett, 2005). AvrRpt2 causes the proteolysis of RIN4; a protein that is required for RPM1 mediated disease resistance (Lim and Kunkel, 2004). The R protein RPS2 monitors the regulation of RIN4. With the elimination of RIN4, RPM1 is not

activated as presence of RIN4 is required for RPM1 disease resistance activation (Mudgett, 2005, Maor and Shirasu, 2005, Lim and Kunkel, 2004).

The Plant Immune System

Mobile defender cells and a somatic adaptive immune system, found in mammals, are lacking in plants (Jones and Dangl, 2006). Despite this, plants are still able to exhibit a response to a foreign invader. Plants not only rely upon the innate immunity of each cell but also on the systemic signals emitted from infection sites (Jones and Dangl, 2006).

Plants have evolved mechanisms to detect pathogen invaders. These are known as pathogen- or microbe associated molecular patterns (PAMPS or MAMPS) (Jones and Dangl, 2006). Some examples of MAMPS are components of the bacterial flagella, fungal chitin, cold-shock proteins, lipopolysaccharides, hairpins, peptidoglycans and elongation factor Tu (Bittel and Robatzek, 2007, Jones and Dangl, 2006, Zhou and Chai, 2008). Host pathogen recognition receptors (PRR) are located on the cell surface and upon MAMPS recognition, they trigger a basal defense response (also known as PAMP-triggered immunity (PTI)) (Jones and Dangl, 2006). The basal defense response that is

triggered by the host pathogen recognition receptors is usually effective at preventing infection. In plants, this basal defense response involves the induction of MAPK signaling cascades, calcium flux, nitric oxide and reactive oxygen species production, the thickening of the cell wall and stress-WRKY transcription factor activation (McCann and Guttman, 2007).

Pathogens have evolved a mechanism to slip past the plants immunity. Many pathogens are capable of deploying effectors that contribute towards the pathogens virulence (Jones and Dangl, 2006). With the process of evolution, both plants and pathogens have developed numerous ways of overcoming the response elicited by the other. The deployed effectors interfere with the signaling pathways that result in the activation of the PTI and result in an effector-triggered susceptibility (ETS) (Jones & Dangl, 2006 McDowell and Simon, 2009). Plants have nucleotide binding (NB) and Leucine rich repeats (LRR) that are capable of recognizing the deployed effectors and in turn help contribute towards the effector-triggered immunity (ETI) (Zhou & Chai, 2008).

The ETI is an amplified and accelerated PTI response, and usually results in a hypersensitive cell death response at the site of pathogen infection (Jones and Dangl, 2006).

Through the process of natural selection, the pathogens are driven to avoid the ETI and this is done by shedding or diversifying the recognized effector gene, or even by acquiring additional effectors that are capable of suppressing the ETI (Jones and Dangl, 2006 Zhou & Chai, 2008).

Type III effectors are the only known proteins in bacterial pathogens that can elicit the ETI (Block et al., 2008, Jones and Dangl, 2006). There is some overlap in the signaling pathways that occur between the ETI and PTI. The direct recognition of an effector by an R protein may lead to the ETI. According to the guard hypothesis however, many R proteins detect the modifications of the host targets made by the specific effectors, and in many cases it is not just the recognition of the presence of effectors (Block et al., 2008 and Jones and Dangl, 2006).

Phytopathogens

Phytopathogenic bacteria infect plants using a multitude of methods. They make use of the numerous secretions systems, among them the type I, II, III, IV, V and VI secretion systems. Despite this, all have in common the goal of getting the pathogen inside the plant cell and making sure the plant defenses are inactivated. For

example, *Erwinia amylovora* that causes fire blight and *Erwinia chrysanthemi* use the Type I Secretion System (Guttman, 2004), whereas *Agrobacterium tumefaciens* and *Xanthomonas campestris* pv. *vesicatoria* use the Type IV Secretion System (Guttman, 2004). The most common genera of gram negative bacterial phytopathogens are *Pseudomonas*, *Xanthomonas*, *Ralstonia*, *Erwinia* and *Pantoea*, and all of these phytopathogens make use of the Type III Secretion System to inject their virulence factors into their host cells (Alfano and Collmer, 1996, Bretz and Hutcheson, 2004, Collmer et al, 2002, Grant et al., 2006, He et al., 2004, Mudgett, 2005).

All pathogens that utilize the Type III Secretion system can be divided into 2 groups based on highly conserved core structural component similarity (Galan and Collmer, 1999). The first group is composed of predicted outer membrane proteins and also includes proteins that have sequence similarity to the secretin family of protein transporters. This group also includes components that have several less conserved lipoproteins (Galan and Collmer, 1999). The other group comprises integral membrane proteins that are comparable to components of the flagellar export apparatus (Galan and Collmer, 1999).

The *Pseudomonas syringae* spp. and *Erwinia* spp. are classified as group I, and *Ralstonia* and *Xanthomonas* spp. are classified as group II. *Pseudomonas syringae* pv. *tomato* is more closely related to the *Yersinia* spp. than it is to the *Xanthomonas* spp. (Preston, 2001). Each strain of bacteria causes a different type of disease. For example, *Pseudomonas syringae* pv. *tomato* causes bacterial speck, *Erwinia carotovora* causes soft rots, and *Ralstonia solanacearum* causes vascular wilt (Collmer et al., 2000).

Pseudomonas syringae

Pseudomonas syringae is a plant-associated bacterium that is found as a harmless symbiont on the surface of leaves. When optimum conditions are present, it is able to cause significant agricultural and economic concern (Sarkar and Guttman, 2003). *Pseudomonas syringae* is a seed borne phytopathogen that survives as a saprophyte in the soil, plant debris and on leaf surfaces. Leaf wetness and cool temperatures of around 13-25°C (Pedley and Martin, 2003) are favorable for the development of disease symptoms such as black leaf spots and dark specks on tomato that become sunken (Pedley and Martin, 2003, Preston, 2001).

More than 50 pathogenic strains or pathovars of *Pseudomonas syringae* have been identified based on their

host specificity (Sarkar and Guttman, 2003). Among them are *Pseudomonas syringae* pathovar *syringae* that causes bacterial brown spots in beans, *Pseudomonas syringae* pathovar *phaseolicola* that causes halo blight on beans, *Pseudomonas syringae* pathovar *maculicola* that causes bacterial leaf spots on cruciferous plants and *Pseudomonas syringae* pathovar *tomato* DC3000 that causes bacterial speck in tomatoes (Buell et al., 2003, Collmer et al., 2000).

Pseudomonas syringae spp. are motile, rod-shaped gram-negative aerobes (Preston, 2001). *Pseudomonas syringae* pathovar *tomato* has been completely sequenced and annotated (Buell et al., 2003), thus making it the most studied of all *Pseudomonas syringae* strains. A reason to why this strain is frequently studied is the ease at which this pathogen is cultured and manipulated using a wide range of molecular genetics and cell biology techniques. Another crucial reason as to why this is a frequently studied strain is due to it being pathogenic to the model plant *Arabidopsis thaliana* (Preston, 2001).

The genome of *Pseudomonas syringae* pathovar *tomato* DC3000 is 6.5 megabases, contains a circular chromosome and 2 plasmids that encode for 5763 open reading frames (Buell et al., 2003). 298 virulence genes have been identified, including various clusters of genes that encode for the 31

confirmed and 19 predicted effector proteins (Buell et al., 2003). Analysis looking for similarity among *Pseudomonas* spp. has shown a high degree of similarity between *Pseudomonas syringae* pathovar tomato DC300 with *Pseudomonas putida* and *Pseudomonas aeruginosa*. However, there are still 1159 genes that are unique to *Pseudomonas syringae* pathovar tomato DC3000, 811 of which lack any known function (Buell et al., 2003).

Type III Secretion System

Many gram-negative bacteria, including *Pseudomonas syringae* use the Type III Secretion System (TTSS) to inject effector proteins into host cells. *Pseudomonas syringae* pathovar tomato DC3000 uses the Hrp pilus to shuttle/inject its virulence factors across the plant cell wall into the plant cytoplasm (Alfano and Collmer 1997, Buttner and Bonas 2002, 2006, Yuan and He 1996). The hrp pilus is more flexible and measures between 6-8nm in diameter by 2µm in length. When compared to the TTSS in animal pathogens, the animal needle is a more stiff structure, roughly around 80nm in length (Bretz and Hutcheson 2004, Galan and Collmer 1999). This is likely due to the structural hindrances that the plant pathogen must deal with; it has a much greater distance to traverse across the thick plant cell wall.

The Type III secretion system pathway is encoded by hrp (HR and pathogenicity) and hrc (HR and conserved) genes. Lindgren et al. first identified Hrp genes in 1986 in *Pseudomonas syringae* pathovar *syringae* (Lindgren et al., 1986). Hrp genes are clustered, spanning up to 41kb of DNA and located on the chromosome (Van Gijsegem et al., 1995). The biochemical functions of these hrp genes are unclear but many show similarity with other known genes. Molecular and biochemical characterization research performed on the hrp genes of numerous *Pseudomonas syringae* strains have provided evidence to show that the hrp genes function in protein secretion and gene regulation (He, 1996).

The hrc proteins direct the secretion of TTSS substrates across the bacterial envelope and some of them are secreted by the TTSS and direct the translocation of effectors through host cell barriers. The designation of Hop (Hrp outer proteins) has been given to effectors that are capable of migrating across the TTSS pathway (Alfano and Collmer, 2004).

Translocation Apparatus

The secretion/translocation apparatus is constructed upon expression of activated hrp genes (Jin et al., 2003, Yuan and He, 1996, Collmer et al., 2000). The hypothesis

that both the type III secretion system and the flagella are related stems from the similarity among the eight *hrc* genes and the flagella assembly genes. Since the discovery of the Hrp pilus in *Pseudomonas syringae* in 1997, similar discoveries have been made in *Ralstonia solanacearum*, *Erwinia amylovora*, *Xanthomonas campestris* and *Sinorhizobium fredii* (He and Jin, 2003, Jin et al., 2003).

The major subunits of the pili are small sized proteins of around 6 to 11kDa that are sometimes referred to as pilins (He and Jin, 2003). HrpA, which is the major subunit of the Hrp pilus, is a 113-amino acid protein (Yuan and He, 1996). HrpA is essential in causing the elicitation of the HR in plants. The C-terminus of the HrpA is responsible for the formation of the pili and for virulence, whereas the N-terminus is not needed in the formation of the filamentous pili (Yuan and He, 1996). It has been shown in many studies that the presence of a functional HrpA protein is required for the secretion of the HrpZ hairpin (Grant et al., 2006, Mudgett, 2005, Yuan and He, 1996).

There is significant difference in sequence, even among the different strains of *Pseudomonas syringae*. Mutants of *Pseudomonas syringae* pv. *tomato* DC300 that contain a *hrpA* deletion mutation, lack the capability to

produce the Hrp pilus and secrete HrpW and AvrPto (Jin, et al., 2003). It has been shown that all plant pathogens secrete hairpin proteins but this is not true in animal pathogens, thus this leads to the suggestion that these hairpin proteins are somehow involved in helping with the penetration of the TTSS pilus through the plant cell wall (He and Jin, 2003, Yuan and He, 1996).

There is mounting evidence that points at the Hrp pilus being an essential part in protein delivery (He and Jin, 2003). The HrpA genes of *Pseudomonas syringae* are required for the extracellular secretion of effector proteins. As shown by He et al., 2003, via an immunogold labeling experiment, the secreted proteins HrpZ, HrpW and AvrPto are colocalized along the length of the Hrp pilus and not found just anywhere in the extracellular space (He et al., 2003).

Type III secretion occurs at the site of the pilus assembly and effector protein are secreted while the pilus is being constructed (Li et al., 2002). Two alternative models have been proposed to explain the localization of the secreted proteins along the length of the pilus. The first model, which is the "conduit" model, states that the effector proteins are secreted as the Hrp pilus grows in length, leaving behind a trail marking the growth of the

pilus (He et al., 2004, Q Jin et al., 2001,). The other model, which is known as the "guiding filament/conveyor model", states that the Hrp pilus carries the effector proteins with it as it grows out of the Type III secretion basal body (He et al., 2004, Li et al., 2002).

Type III Secretion System in Phytopathogens

In phytopathogens the TTSS is known as the Hrp system (hypersensitive response and pathogenicity). The nature of the name comes from the Hypersensitive Response (HR), which it causes in plants (Alfano and Collmer, 1996, Bretz and Hutcheson, 2004, Collmer et al., 2000, Collmer et al., 2002, He, 1996). The TTSS has many unique features, among which specific chaperones are required for the secretion of effector proteins (Greenberg and Vinatzer, 2003, He et al., 2004). Another unique feature is that the TTSS consists of two parts, a base and the filamentous appendage, which in animal pathogens are called the needle and in plant pathogens are called the pilus (Alfano and Collmer, 1996, Alfano and Collmer, 1997, Bretz and Hutcheson, 2004, Galan and Collmer, 1999, He et al., 2004, Romantschuk et al., 2001).

Hairpins

All plant pathogens secrete proteins that are part of the hairpin family (Alfano and Collmer, 1996). There are no known mammalian pathogens that secrete these hairpins. Hairpins are hydrophilic, heat-stable acidic proteins, rich in glycine but cysteine-free that are secreted when the Hrp system is expressed (Li, 2007, Alfano and Collmer, 1996). When present at high concentration in the plant apoplast, the hairpin proteins elicit the HR. They travel through the secretion apparatus but are not injected into the host cell. They are however released into the apoplastic space. Hairpin proteins have also been suggested to be involved in assisting the Hrp pilus in penetrating the plant cell wall (Alfano and Collmer, 1996, He, 2004).

Chaperones

Type III effector proteins require the use of chaperone proteins to be translocated into the host cell. These proteins are acidic in nature, rich in leucine and are roughly about 170 amino acids in length, and contain an amphipathic α helix near the C-terminus (Jin et al., 2003). At present, there are three known classes of type III secretion chaperones (Parsot et al., 2003, Jin et al., 2003). Class I chaperones are involved in the binding of

the effector protein at the chaperone binding domain (CBD), which is located within the first 100 amino acids of the effector protein (Akeda and Galan, 2005). Class I chaperones are further divided into 2 classes, Class IA and Class IB (Parsot et al., 2003, Kabisch et al., 2005). Class IA chaperones interact with either one or several homologous effectors and are encoded next to their interaction partners, whereas Class IB chaperones are capable of binding with a wide range of effectors (Kabisch et al., 2005, Parsot et al., 2003). Chaperones classified under Class II are involved in the translocation apparatus formation. Class III chaperones are chaperones of the flagellar system (Parsot et al., 2003). Genes that are usually found on pathogenicity islands encode the type III secretion system chaperones.

Chaperones are required for translocation and secretion of the effectors and sometimes function to protect the effectors from proteolysis or aggregation in the bacterial cytoplasm (Jin et al., 2003). They however are not required for the secretion of proteins produced due to conditions of down regulation activity (Jin et al., 2003, Parsot et al., 2003). Chaperones switch from their bound state where they are bound to their specific effector to a free state upon activation of secretion (Parsot et

al., 2003). Upon secretion, chaperones release their bound effectors, as they themselves are not secreted into the host cell, and must remain within the bacterial cytoplasm (Akeda and Galan, 2005).

The type III secretion chaperones play numerous roles. They play a role as anti-folding factors, where they function to maintain the effectors they associate with in an unfolded state (Kabisch et al., 2005, Parsot et al., 2003). The translocation channel is only about 2- 2.5nm in diameter and thus is too small to allow a fully folded effector to pass through it. Therefore, in order to pass thru the channel, the effector proteins have to be maintained in a semi or completely unfolded state (Parsot et al., 2003, Kabisch et al., 2005).

Besides functioning as anti-folding factors, the chaperones also function as secretion signals and as stability factors (Parsot et al., 2003). The effector protein YopE of *Yersinia*, in the absence of its chaperone SycE, is unstable and undergoes rapid degradation (Mota et al., 2005). In the plant pathogen *Erwinia amylovora*, chaperone DspF is required for the stability of DspE (He, 2004). A recent study done in *Pseudomonas syringae* showed that the chaperones protect their respective effectors from Lon-associated degradation (Losada and Hutcheson, 2005).

Most of the type III secretion system plant pathogenic bacteria chaperones were discovered in *Erwinia amylovora* and *Pseudomonas syringae*. These chaperones are specific for one or several individual effectors (Parsot et al., 2003, Badel et al., 2003). HrpG however, is the exception to this. HrpG binds and inhibits the cytosolic HrpV protein, which is a negative regulator of hrp gene expression. Therefore HrpG is the first of the type III secretion chaperones in plant pathogenic bacteria to have a regulatory role (Buttner, 2006). At present, not much is known about the chaperones of *Pseudomonas syringae* pv. *tomato* DC3000. ShcS1 and ShcO1 are the two chaperones of *Pseudomonas syringae* pv. *tomato* DC3000 that have been most studied.

Hrp Outer Proteins

Type III effector proteins are proteins that are secreted via the type III secretion system into the host plant in order to overcome the host's defenses. In the case of plant pathogens, the effectors function within the plant cell and many appear to be post-transcriptionally modified by the host enzymes. *Pseudomonas syringae* pv. *tomato* DC3000 has over 30 effector proteins that are the focus of many studies (Buell et al., 2003, Grant, 2006). Among them are

AvrPtoB or also known as HopAB2, HopAO1 and HopM1, HopQ1-1, HopU1 and many others. I will briefly talk about AvrPtoB, HopAO1 and HopM1, with the focus of this study being HopM1.

AvrPtoB

AvrPtoB or also widely known as HopAB2, is a 553 amino acid protein (Abramovitch et al., 2003, Xiao et al., 2007). It is a widely conserved effector protein found in numerous *Pseudomonas syringae* strains as well as in *Xanthomonas spp.* and *Erwinia spp.* It was first identified based on its ability to trigger immunity on resistant tomato plants expressing Pto and Prf and was therefore recognized as an avirulence protein (Abramovitch, et al., 2003, Xiao et al., 2007).

AvrPtoB has been determined to have 2 distinct avirulence factors that are found in the N-terminal region (Abramovitch et al., 2003). The first one is contained within amino acid 1-307, is recognized by Pto Kinase, an R protein, and the second one which is contained within amino acids 1-187, is recognized by the R protein Fen kinase. The C-terminal region of AvrPtoB is an E3 ligase which ubiquitinates the host R protein Fen, thus promoting its degradation and causes disease susceptibility (Abramovitch et al., 2003, Xiao et al., 2007, Xiang et al., 2008).

AvrPtoB₁₋₃₀₇ in susceptible tomato plants is sufficient in promoting bacterial growth and enhancing disease symptoms that is associated with an increase in ethylene production (Xiao et al., 2007). In *Arabidopsis*, it has been shown that AvrPtoB₁₋₃₈₇ is required for the suppression of pathogen associated molecular pattern triggered immunity. Due to the fact that the activity of AvrPtoB₁₋₃₀₇, AvrPtoB₁₋₃₈₇, and full length AvrPtoB is indistinguishable from each other, this indicates that residues 308-553 have a redundant phenotype or lacks virulence activity in tomatoes (Xiao et al., 2007).

HopA01

HopA01 (also known as HopPtoD2) is a 468 amino acid protein (Alfano and Collmer, 1996, Brentz et al., 2003, Grant et al., 2006, Underwood et al., 2007). The C terminal of HopA01 contains a protein tyrosine phosphatase (PTP) domain. This effector shares some homology with the effector protein SptP that is found in *Salmonella* and the effector protein YopH of *Yersinia*. The N terminal domain of HopA01 shows some resemblance to that of another effector, HopPtoD that has no predicted protein function (Grant et al., 2006, Dean, 2011).

It has been shown that the 321 amino acid terminal domain alone is not effective in eliciting or affecting plant defense responses, and that therefore the carboxyl terminal domain is responsible for the activity of this effector (Bretz et al., 2003, Dean, 2011). The exact role of HopA01 in plants remains unclear, but it plays an important role in *Pseudomonas* host interactions. As suggested by Nurnberger and Schell, 2001 and Zhang and Klessig, 2001, a possible target for the PTP activity of HopA01 is one or more of the MAPK dependent signal transduction pathways that controls defense responses.

In susceptible hosts such as *Arabidopsis* and tomato, mutants of HopA01 show a reduced growth rate (Mudgett, 2005). In resistant hosts, hydrogen peroxide production and PR1 expression is decreased, and the HR initiation is blocked. HopA01 also suppresses the HR that is induced in *Nicotiana benthamiana*.

HopM1

HopM1 (also known as HopPtoM) is a 712 amino acid protein (Block et al., 2008, Block et al., 2011). The effector protein HopM1 is conserved among all strains of *Pseudomonas syringae*. There are no known domains present in HopM1 nor does it share any homology with any other known

proteins. HopM1 has been shown to interfere with vesicle trafficking (Nomura et al., 2006, Nomura et al., 2011, Thordal-Christensen, 2009). As vesicle transport is important for the export of defense compounds to the cell wall and apoplast, its disruption would most certainly impair cell wall based defenses (Block et al., 2008, Thordal-Christensen, 2009).

HopM1 is found in the endomembrane fraction of plant cells in *Arabidopsis*. HopM1 targets the ARF-GEF AtMIN7 (adenosine diphosphate ribosylation factor guanine nucleotide exchange factor HopM interactor 7), for degradation via the hosts' 26S proteasome (Nomura et al., 2006, Nomura et al., 2011). Organelles of the endomembrane system are all part of the secretory pathway and consist of the nuclear envelope, Endoplasmic reticulum, Golgi apparatus, lysosomes, vacuoles, vesicles and cell membranes.

Due to the fact that HopM1 does not share any homology with E3 ligases, it is highly possible that it serves as a linker between AtMIN7 and the proteasome degradation machinery of the host (Citovsky et al., 2009, Nomura et al., 2006, Nomura et al., 2011). Brefeldin A, an inhibitor of exocytosis, is capable of mimicking the effects of HopM1, by inhibiting the GEF activity of the Sec7 protein

family which AtMIN7 is a member of (Nomura et al., 2006), thus supporting the fact that AtMIN7 plays a role in defense component secretion (Thordal-Christensen, 2009).

In a study done by Nomura et al., in 2006, they were able to show that a HopM1 mutant containing amino acids 101-712 was able to partially restore the bacterial multiplication and chlorotic symptoms of the conserved effector locus mutant in *Arabidopsis*. They were also able to show that mutants containing just amino acid 100-200 and 100-300 exerted a dominant negative effect on full length HopM1 function. These results hence lead to the conclusion that the N-terminal 200 to 300 amino acids are able to function as an independent domain *in vivo*, interfering with the virulence function of the full length HopM1 (Nomura et al., 2006).

CHAPTER 2

CREATION OF A YEAST MODEL SYSTEM TO EXPRESS *PSEUDOMONAS SYRINGAE* PATHOVAR TOMATO DC300 EFFECTOR PROTEINS

Introduction

The majority of gram-negative bacterial pathogens make use of the type III secretion system to inject their effector proteins, thus causing disease in their animal or plant hosts. These effector proteins cause disease by either attacking the host cells innate immune system, by altering the vesicle trafficking pathway or by modifying the cytoskeleton and membranes (Nomura et al., 2006). The effector proteins are translocated directly across the cell wall into the cytoplasm via the type III secretion system injectisome, thus avoiding the basal resistance mechanism of the cell.

Pseudomonas syringae is one of many gram-negative phytopathogens that utilize this specific secretion system. The Type III secretion system in *Pseudomonas syringae* is encoded by the hypersensitive response and pathogenicity (*hrp*) and the hypersensitive response and conserved (*hrc*) genes of the Hrp pathogenicity islands (Munkvold et al., 2008, Guo et al., 2009). The effector proteins that are injected into the host cell cause disease in susceptible

plants mainly by suppressing the plant innate immunity. The effectors injected into resistant plants are detected by the plant resistant (R) proteins, thus activating the effector triggered immunity (ETI), which triggers the activation of the hypersensitive response (HR); a programmed cell death (Guo et al., 2009, Munkvold et al., 2008).

Depending on the phenotype displayed, the effector proteins are either designated as Hrp dependent outer proteins (Hops) or avirulence (Avr) proteins. All the effector proteins that are secreted/translocated via the type III secretion system display a Hop phenotype and some also exhibit an Avr phenotype, depending on if the effector or its activity is recognized by the host cells resistance (R) genes, which often results in the elicitation of the hypersensitive response (Schechter et al., 2006).

There are many different strains of this phytopathogen, with one being *Pseudomonas syringae* pathovar tomato DC3000. This pathogen causes bacterial speck in tomatoes, the model system *Arabidopsis thaliana* and *Nicotiana benthamiana*. This particular phytopathogen translocates well over 40 different effector proteins into its host cytoplasm, thus wrecking havoc to the hosts' defense mechanism. The one reason behind this phytopathogen

being well studied is that its entire genome has been sequenced (Buell et al., 2003) and many of its effector proteins are conserved among the different strains.

Of the 40 effector proteins that are injected into the cytoplasm, one that is of interest to us is effector protein HopM1. HopM1 is conserved among all strains of *Pseudomonas syringae* and it has been the focus of different groups and here, we intend to present our results that have proven to contradict published data. HopM1 is a 712 amino acid with no known domains (Nomura et al., 2006). It localizes to the plant endomembrane as described by Nomura et al., 2006. HopM1 targets AtMIN7, a putative adenosine-diphosphate ribosylation factor-guanine nucleotide exchange factor that is involved in intracellular vesicle trafficking in *Arabidopsis thaliana*. HopM1 mediates the 26S proteasome-dependent degradation of AtMIN7, a key player in the initiation of vesicle trafficking (Nomura et al., 2006).

The use of *Saccharomyces cerevisiae* as a model system has been well established in accordance with studying the effects of heterologously expressed proteins including effector proteins. Due to yeast lacking the R protein surveillance system that causes cell death during the normal defense response in typical pathogen interactions,

we have been able to utilize yeast in accordance with our goals to study the virulence effect of the effectors on yeast in the absence of the hypersensitive response. We have been able to utilize the inducible *GAL1* promoter in our efforts in light of research done by Liu et al., 1992, which demonstrated that a severe inhibition phenotype seen in yeast under a *GAL1* promoter is very small and more than likely any inhibitory effect seen could be due to the expression of the effectors (Munkvold et al., 2008).

In a paper published by Lesser et al., 2001, it is shown that YopE, a GTPase activating protein, is cytotoxic and disrupts the actin filaments in cells. Since then, many groups have used yeast to examine the phenotypes and the disruption of cellular processes by bacterial effector proteins from both mammalian and plant pathogens. With this idea in mind, we are hoping that we would be able to shed some light on the cellular pathways that are disrupted in yeast due to the effects of HopM1.

In a paper published by Munkvold et al., 2008, results were presented showing that one of the effector proteins of *Pseudomonas syringae* pv. *tomato* DC3000, HopM1 has no effect in yeast. In their study, Munkvold et al., 2008, looked at the effect HopM1 had on *Saccharomyces cerevisiae* at 30°C. Here we are presenting our data showing that HopM1 has a

phenotype in yeast and localizes to the mitochondria and organelles or vesicles of the secretory pathway, in accordance with results shown by Nomura et al., 2006, which shows that HopM1 localizes to the plant endomembrane when expressed in *Arabidopsis thaliana*.

Materials and Methods

Media

LB (Luria-Bertani) media with appropriate antibiotic was used for growth of bacterial culture, and has been previously described (Sambrook et al., 1989).

Media for yeast growth such as YPD, YPG, S-ura, S-ura-leu (with and without glycerol), Sgal-ura, Sgal-ura-leu (with and without glycerol), have been previously described (Guthrie and Fink, 1991).

Plasmid Construction

Plasmids used in this study are listed in Table 1. Plasmid pJG485 was constructed as described by Nejedlik et al., 2004. Plasmid p416 GAL1 (Mumberg et al., 1994) that contains the *URA3* auxotrophic marker and an inducible galactose promoter *GAL1* was digested to liberate the *GAL1* vector and was ligated with the GATEWAY™ fragment of pYES

Dest52, forming pJG485. Plasmid pJG484 was constructed in a similar way, except it contained the *LEU2* auxotrophic marker.

HopM1, HopA01 and HopAF1 were PCR amplified from *Pseudomonas syringae* pv. *tomato* DC3000 genomic DNA obtained from ATCC using the N-terminal oligonucleotide attB1- HopM1 (5' - GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGATCAGTTCGCGGATCG- 3') and the C-terminal oligonucleotide attB2- HopM1 (5'- GGGGACCACTTTGTACAAGAAAGCTGGGTACGCGGGTCAAGCAAGCCCT- 3'), N-terminal oligonucleotide attB1- HopA01 (5' - GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGAATCCCCTGCAACCTATTC- 3') and the C-terminal oligonucleotide attB2- HopA01 (5' - GGGGACCACTTTGTACAAGAAAGCTGGGTCCTGTCCTCTAGGAATGGC- 3'), and N-terminal oligonucleotide attB1- HopAF1 (5' - GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGGGGCTATGTATTTCAA- 3') and the C-terminal oligonucleotide attB2- HopAF1 (5' - GGGGACCACTTTGTACAAGAAAGCTGGGTCTTGTGCGACCAGATGTTTTATG- 3') respectively. The resulting PCR fragments were combined with pDONOR201 and allowed to recombine using the Invitrogen GATEWAY™ BP reaction. The plasmid resulting for the BP reaction, pJB1, pVR10 and pVR12 contained HopM1, HopA01 and HopAF1 respectively with the flanking attL1 and attL2 sequences. The plasmids were sequenced to determine sequence verification.

The GATEWAY™ LR reaction was used in order to create plasmid pJB3, pVR20 and pVR21, which resulted from the recombination of plasmid pJB1 and pJG485, pVR18 with pJG485, and pVR19 with pJG485 respectively. The GATEWAY™ LR reaction was also used to create plasmid pJB2, which resulted from the recombination of pJB1 and pJG484. Plasmids pJB2, pJB3, pVR20 and pVR21 all contained the V5-6xHis construct that would be expressed upon induction with galactose.

Plasmid	Yeast	Bacteria	Reference
pDONOR201		f1 Kan ^R attP1 ccdB Cm ^R attP2	Invitrogen
pDONOR221		f1 Kan ^R attP1 ccdB Cm ^R attP2	Invitrogen
pJG484	<i>CEN6 ARH4 LEU2</i> P _{GAL1} -(attR1 Cm ^R ccdB attR2)-V5-6xHis-CYC1 _{term}	f1 bla	[5]
pJG485	<i>CEN6 ARSH4 URA3</i> P _{GAL1} -(attR1 Cm ^R ccdB attR2)-V5-6xHis-CYC1 _{term}	f1 bla	[4]
pJB1	attL1 <i>hopM1</i> attL2	f1 Kan ^R	This study
pJB2	<i>CEN6 ARSH4 LEU2</i> P _{GAL1} -(attB1 <i>hopM1</i> attB2)- V5-6xHis-CYC1 _{term}	f1 bla	This study
pJB3	<i>CEN6 ARSH4 URA3</i> P _{GAL1} -(attB1 <i>hopM1</i> attB2)- V5-6xHis-CYC1 _{term}	f1 bla	This study
pVR18	attL1 <i>hopA01</i> attL2	f1 Kan ^R	This study
pVR19	attL1 <i>hopAF1</i> attL2	f1 Kan ^R	This study
pVR20	<i>CEN6 ARSH4 URA3</i> P _{GAL1} -(attB1 <i>hopA01</i> attB2)-V5-6xHis-CYC1 _{term}	f1 bla	This study
pVR21	<i>CEN6 ARSH4 URA3</i> P _{GAL1} -(attB1 <i>hopAF1</i> attB2)-V5-6xHis-CYC1 _{term}	f1 bla	This study

Table 1. Plasmids used in this study.

Yeast Strains

Strain	Chromosomal
JGY4	MAT α lys2-801 his3-200 leu2-3, 112 ura3-52
JGY709	MATa/MAT α ade2-101/ADE2 LYS2/lys2-801 his3-200/his3-200 leu2-3, 112/leu2-3, 112 ura3-52/ura3-52

Table 2. Yeast strains used in this study.

Lithium Acetate Yeast Transformation

Each yeast strain was transformed as described (Gietz and Schiestl, 1991). The night before transformation, the different strains of yeast were inoculated into 25ml of appropriate media and allowed to grow in a water bath at 30°C. The next day, the cultures were diluted back into fresh media and once again were allowed to grow in a water bath at 30°C until it reached log phase. The cultures were then centrifuged and resuspended in 5ml of dH₂O. The cultures were once again centrifuges and resuspended in 1.5ml of 1X LiOAc and 1X TE at pH 7.5, and incubated in a spinning wheel incubator for 1 hour at 30°C. 20 μ l of heated denatured sheared salmon sperm DNA and 5 μ l of transforming DNA were added to the tubes and returned to the incubator for 30 minutes. Next, 1.2ml of sterile 40%PEG, 1X LiOAc and 1X TE at pH 7.5 were added to the tubes and returned to the

incubator for 30 minutes. The tubes were then heat shocked for 15 minutes at 42°C. The cultures were then centrifuged in a Labnet Spectrafuge 16M Microcentrifuge at 3000rpm and washed with 1ml sterile 1X TE at pH 7.5. The cultures were plated onto appropriate selective dropout media and allowed to grow at room temperature for 5 days.

Western Blotting

Western Blotting was performed as previously described by Nejedlik et al., 2004. Yeast strains were grown to about mid-log phase in SD-Ura minimal media with raffinose as the sole carbon source. At time point zero, the cells were induced with Galactose to a final concentration of 2%. Aliquots were taken at time point 1, 3, 24 and 48, and the extracts were prepared as described by Kahana et al., 1998. 100µg of the extracts from each time point were separated on an 8% SDS polyacrylamide gel (Laemmli, 1970) and transferred to a PVDF membrane at 30V overnight (Sambrook et al., 1989). To achieve western immunoblotting, the membrane was blocked with 0.2% I-Block (Tropix) in PBS. This was followed by the application of anti-V5 antibody (Invitrogen) at 1:200 dilution. Visualization of the HopM1-V5 protein was accomplished using the ECL Western Blotting Analysis Kit from Amersham.

Serial Dilution Replica Plating

Serial dilution replica plating was performed as described below. Transformants of HopM1 and the control vector pJG485 were grown overnight in S-ura-leu and S-ura media containing 2% raffinose at 21°C. The next day, the transformants were plated onto S-ura-leu, S-ura, Sgal-ura, Sgal-ura-leu and YPD solid media via replica plating. The plates were maintained at room temperature and allowed to grow for about 5 days.

Titer Assay

A titer assay was performed as previously described by Nejedlik et al., 2004. The respective expression vector containing HopM1, pJB3 and an empty vector pJG485 as a control were first transformed into JGY4 haploid strain of yeast. These transformants were then grown up in S-ura medium respectively with 2% raffinose as the carbon source and allowed to grow overnight at 21°C. The next day, the cultures were diluted back into fresh medium with appropriate amount of raffinose and allowed to grow to mid log phase, upon which HopM1 expression was induced via the addition of 2% galactose. 1ml samples were taken at each time point after the flocculence of the cultures were measured and recorded. The samples were then sonicated for

10s at 3.5W using a Fisher Model 50 Sonic Dismembrator. A 10 fold serial dilution was performed on each sample and 100 μ l of each dilution was plated onto SD-ura media. The plates were left at room temperature to grow for about 1 week, after which the number of colony forming units per ml was determined.

Hop Immunofluorescence

Immunofluorescence was performed as previously described by Geiser et al., 1997, Hoyt et al., 1997 and Kahana et al., 1998). Aliquots of 100 μ l of sonicated samples of cells from each time point were fixed in 3.7% formaldehyde at room temperature for 2 hours. The samples were digested with 50 μ g/ml of Zymolyase 100T, and then dissolved in 1.2M Sorbitol, 25mM β -mercaptoethanol, and 100mM KPO₄ for 1 hour at 30°C to dissolve the cell wall. The samples were then washed with 0.04%PBS-BSA and applied to polylysine-coated slides. Ideally, to visualize the Hops, mouse monoclonal anti-V5 antibody was diluted 1:800 in 0.04%PBS-BSA and allowed to incubate for 2 hours at room temperature. The samples were once again washed 3 times with 0.04%PBS-BSA and the goat anti-mouse CY2 (1:100) was applied to the samples and allowed to incubate for 2 hours at room temperature. DAPI (4', 6- Diamidino-2-phenylindole

dihydrochloride from Sigma-Aldrich) was used to stain the DNA and was applied to the samples for 3 minutes at room temperature. Mounting solution was prepared following the protocol outlined by Pringle et al., 1991. The slides were observed using a Leica DM5500B microscope with a Q-Imaging Retiga Exi 1394 fast camera.

Mitochondrial Staining

Fluorescent staining of mitochondria were performed using Mitotracker Red CMXRos (Invitrogen). Cultures were grown in appropriate media and conditions for at least two generations in log-phase. Mitotracker Red CMXRos (1mM in DMSO) was added to the culture 30 minutes prior to addition of galactose ($t = 0$ hr.) to a final concentration of 0.4uM. Cells were prepared as above for immunofluorescence and imaged with a Leica TX2 filter set.

Yeast Cellular Fractionation

Yeast Cellular Fractionation was performed as described by Reider and Emr, 2000, Zinser and Daum, 1995 and Wiederhold et al., 2010, with modifications as listed below. Yeast strains were grown overnight in 100ml YPD. The overnight cultures were then harvested and centrifuged in a Sorvall RC 5B Plus centrifuge using a SS-34 rotor for 5

minutes at 500g. The resulting cell pellet was washed with 100mM Sodium Azide and 50mM Tris-HCl pH 7.5 and centrifuged again. The cell pellet was incubated with 100mM EDTA, 0.5% 2-mercaptoethanol and 10mM Tris-HCl pH7.5 at 30°C for 20 minutes. After 20 minutes, a cell pellet was obtained via centrifugation for 5 minutes at 500g, after which it was resuspended in S Buffer (1.2M Sorbitol, 0.5mM MgCl₂ and 40mM HEPES pH7.5). The cells were then converted to spheroplasts with the addition of 50U/OD600 Zymolyase 100T and allowed to incubate for 90 minutes at 30°C. The spheroplasts are washed once in S Buffer and suspended in Lysis Buffer (0.2M Sorbitol, 1mM EDTA, 50mM Tris-HCl pH7.5). A Dounce Homogenizer is then used to lyse the spheroplasts. A sample is collected from the cell lysate and labeled Sample 1. The remaining cell lysate is centrifuged in a Beckman Ultracentrifuge Optima XL-100K using a swing bucket rotor (Beckman SW 55Ti) for 5000g for 10 minutes at 4°C. The resulting pellet is labeled P5000 and the resulting supernatant is labeled S5000. The supernatant is further centrifuged at 20,000g for 1 hour to yield the P20,000 pellet and the S20,000 supernatant. The S20,000 supernatant is further centrifuged for 1 hour at 300,000g to yield the P300,000 pellet and the S300,000 supernatant.

Sucrose Density Gradient Centrifugation

Density sucrose gradients were used to isolate different organelles. A sucrose step gradient using 1.5M Sucrose and 1.2M Sucrose Solutions was prepared using a peristaltic pump to layer the 1.2M Sucrose Solution on top of the 1.5M Sucrose Solution. After the gradients were set up, 200 μ l of the samples resulting from the yeast fractionation protocol were layered over the top of the gradients. The gradients were centrifuged in a Beckman Ultracentrifuge Optima XL-100K using a swing bucket (Beckman SW 55Ti) at 85,000g for 1 hour. Once the gradients were centrifuged, they were carefully removed from the swing buckets and the visible bands present in the different layers of the step gradients were collected and analyzed via Western Blot.

Creation of a Petite Yeast Strain

A petite yeast strain was created as described by Guthrie and Fink, 1991 with modification. Yeast strain JGY4 was grown to saturation in minimal medium containing 2% glucose and 25 μ g/ml Ethidium Bromide. A second culture is started from the first culture and allowed to grow to saturation. From here, a small amount of culture is plated

onto YPD and essentially every colony that grows will be ρ^0 .

Results

Construction of a *Pseudomonas syringae* Effector Model System

There are significant similarities that exist among *Saccharomyces cerevisiae* proteins and mammalian signal transduction pathways, cell cycle pathways and the proteins involved in the construction and modifying of the cytoskeleton (Nejedlik et al., 2004).

To be able to test our model system, we created yeast plasmids pJB2 and pJB3, which contains *Pseudomonas syringae* pv. *tomato* DC3000 *hopM1* that is under the control of *GAL1*, an inducible yeast promoter, and each plasmid contained either the *URA3* or *LEU2* auxotrophic marker. Fused to the 3' end of *hopM1* is DNA that encodes for the V5 epitope and six copies of the histidine affinity purification tag. We also created plasmid pVR20 and pVR21 that contains *Pseudomonas syringae* pv. *tomato* DC3000 *hopAO1* and *hopAF1* respectively. We used pJG484 and pJG485, which were lacking *hopM1* but containing all other plasmid sequences as control plasmids. All plasmids were transformed into the wild-type yeast

strain JGY4 and selected on media lacking uracil and leucine respectively in order to isolate a strain containing each plasmid.

HopM1 is Produced in Yeast

To determine if the model system was working as expected and expressing the effector protein, a western blot was performed. The yeast strain JGY4 containing plasmid pJB2 and pJB3 was grown to mid-log phase in minimal selective media. Effector protein expression was induced and aliquots were taken at numerous time points and processed. As shown in Figure 1, you begin to see the expression of HopM1 beginning at hour 3 after induction and minimal expression is observed at hour 24.

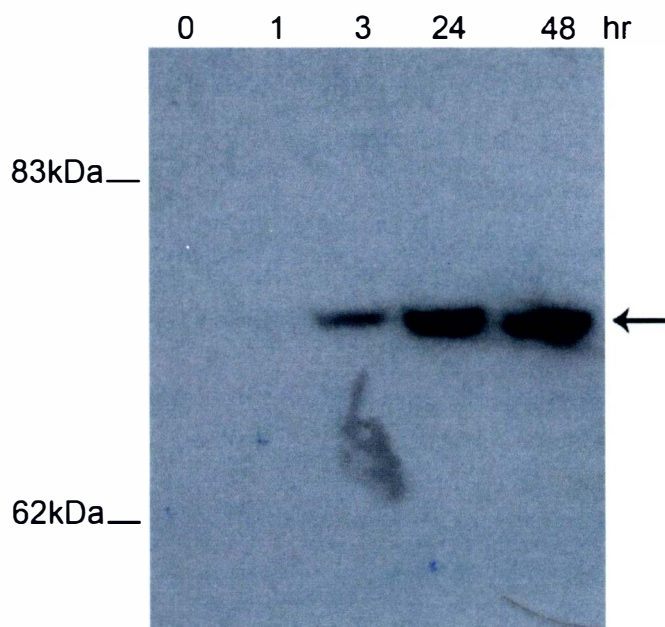


Figure 1. HopM1 is produced in yeast. Yeast strain JGY4 containing pJB2 and pJB3 was induced with galactose for 48 hours. Aliquots were taken and protein extracts prepared. The level of HopM1-V5 in un-induced and induced cultures were determined by immunoblotting. Molecular weight marker is shown. Arrow indicates the expected molecular weight (75kDa) of HopM1 protein.

Expression of *Pseudomonas syringae* Effectors in Yeast

In order to determine if the expression of our numerous plasmids are capable of disrupting the growth of yeast, we compared the growth of the yeast strains containing the different plasmids to one that contained the control plasmid. Yeast strains containing the different plasmids were replica plated onto SD-ura, Sgal-ura, SD-ura-leu and Sgal-ura-leu media and allowed to grow at 21°C for 4 days. As shown in figure 2, the yeast strain containing the

control plasmid and HopAO1 and HopAF1 were able to grow at all conditions and produced colonies across all dilutions. As shown in figure 3, the strain containing HopM1 however, was not able to grow on the Sgal-ura-leu plates at 21°C.

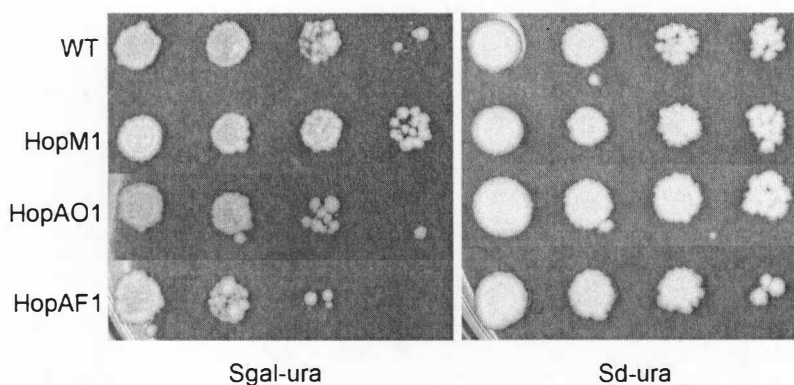


Figure 2. HopAO1 and HopAF1 are not lethal. Yeast strain JGY4 containing control (pJG485), HopM1 (pJB3), HopAO1 (pVR20) and HopAF1 (pVR21) were replica plated onto SD (SD-ura) or Sgal (Sgal-ura) medium and incubated at 21°C for 4 days. Each spot of cells is a 40-fold dilution of the cells in the previous spot.

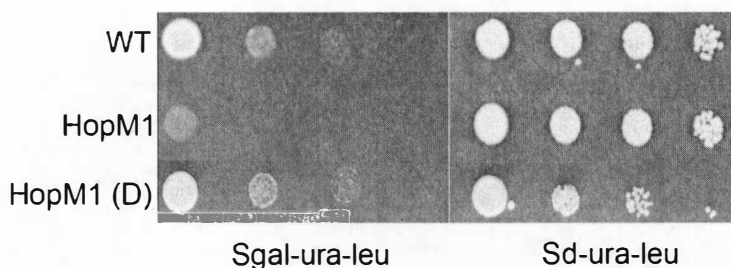


Figure 3. HopM1 is lethal in yeast. Yeast strain JGY4 containing control (pJG4845), HopM1 (pJB2 and pJB3), plasmids were replica plated onto SD (SD-ura-leu) or Sgal (Sgal-ura-leu) medium and incubated at 21°C for 4 days. Each spot of cells is a 40-fold dilution of the cells in the previous spot.

While working on the expression of these three effector proteins, Munkvold et al., 2008, published results on similar work done in yeast with effectors. Their results show that HopM1 is not inhibitory to yeast growth at 30°C, but in our hands, we have been able to show that HopM1 is inhibitory to yeast growth at 21°C. With these results in hand, we set out to look at the effects of HopM1 more extensively.

Titer Assay Of HopM1

To determine if HopM1 expression is cytotoxic or cytostatic to yeast, we measured the strain looking at the number of cells able to grow on solid dextrose media after being removed from galactose induction. The yeast strain containing the control plasmid increased steadily over the course of the 48 hour experiment. The number of cells able to grow in the yeast strain expressing HopM1 slowly began to decrease 7 hours after galactose induction. At hour 24, when the maximum amount of HopM1 was observed by western blot analysis, there were about 91% of viable cells and this number continued to decrease up to hour 48 with only 37.5% viable cells remaining. Refer to Figure 4 for graph.

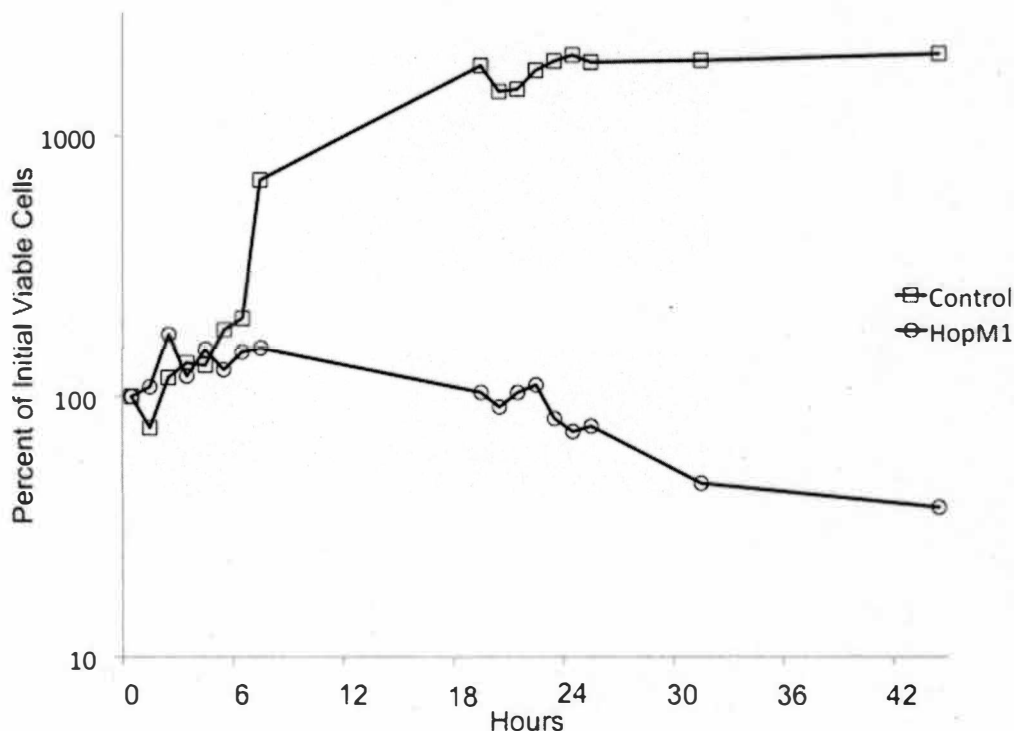


Figure 4. Examination of cell viability of yeast strain containing HopM1. Yeast strain JGY4 containing HopM1 (pJB2 and pJB3) or control plasmid (pJG484 and pJG485) were grown in selective medium containing 2% raffinose. 2% galactose was added to the medium at the zero time point to induce HopM1 production. 1ml aliquots were taken at each time point, sonicated and serially diluted onto SD-ura-leu plates. Each curve is a representative of three different trials for each strain.

Expression of HopM1 is Lethal to Yeast at 21°C

In order to understand the results that were seen by Munkvold et al., 2008, we wanted to examine the effect of HopM1 expression in yeast. This was done by comparing strains containing the HopM1 plasmids with a strain containing the control plasmids. The strains were replica

plated onto Sgal-ura-leu medium and allowed to grow at 21°C, 30°C and 37°C for 4 days. Figure 5 shows the results of this replica plating. The strain containing the control plasmid grew as expected at all temperatures, producing colonies at all dilutions and on all selection media. The strain containing the HopM1 plasmid however, did not grow on the Sgal-ura-leu plates at 21°C, but was able to grow on the Sgal-ura-leu plates at 30°C and 37°C. Our results are consistent with the results presented by Munkvold *et al.*, 2008, at 30°C and 37°C, in which they showed that HopM1 was able to grow on the Sgal-ura-leu selection media at 30°C. Our results show an unappreciated finding in that expression of HopM1 in yeast kills at 21°C but not at 30°C and 37°C.

We examined the expression of HopM1 in haploid (JGY4) and diploid (JGY709) yeast strains. Two HopM1 expressing plasmids (pJB2 and pJB3) were transformed into the respective strains, replica plated onto Sd-ura-leu and Sgal-ura-leu medium and allowed to grow at 21°C for 4 days. Figure 6 shows the results from this replica plating. The strain containing the control plasmids grew as expected, producing colonies across all dilutions. The diploid yeast strain containing the HopM1 plasmids, were able to grow on both Sd-ura-leu and Sgal-ura-leu, as compared to the

haploid yeast strain that was able to grow on only Sd-ura-leu as shown earlier. We do not know why the diploid yeast strain expressing both HopM1 plasmids is able to grow but it could possibly be due to the difference in growth or polarity of the diploid.

To further examine the expression pattern of HopM1 at these different temperatures, we examined the protein expression of HopM1 at multiple growth temperatures.

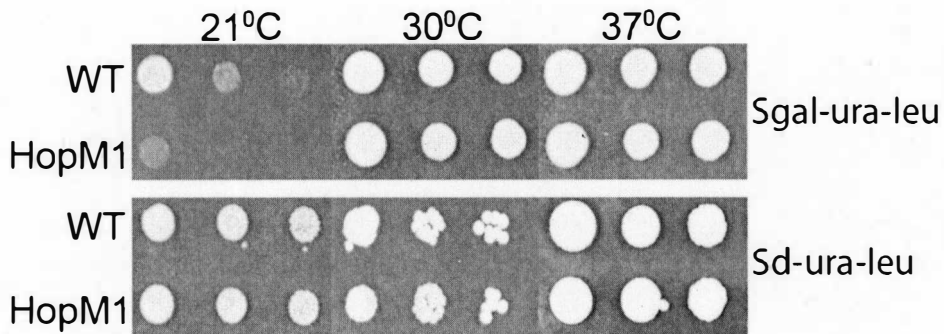


Figure 5. HopM1 is lethal in yeast at 21°C. Yeast strain JGY4 containing control (pJG4845 and pJG484) and HopM1 (pJB2 and pJB3) plasmids were replica plated onto SD (SD-ura-leu) or Sgal (Sgal-ura-leu) medium and incubated at 21°C, 30°C and 37°C for 4 days. Each spot of cells is a 40-fold dilution of the cells in the previous spot.

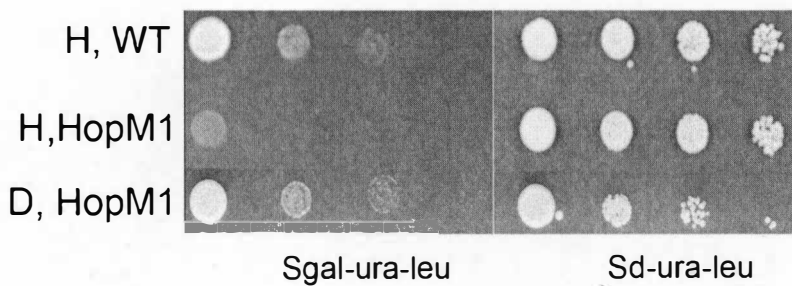


Figure 6. Effects of HopM1 in haploid and diploid yeast. Yeast strain JGY4 (H) and JGY709 (D) containing control (pJG484 and pJG485) and HopM1 (pJB2 and pJB3) plasmids were replica plated onto SD (SD-ura-leu) or Sgal (Sgal-ura-leu) medium and incubated at 21°C for 4 days. Each spot of cells is a 40-fold dilution of cells in the previous spot.

Expression of HopM1 at 21°C, 30°C and 37°C

To further understand the results seen from serial dilution at the different temperatures (figure 5), we performed western blot analysis, looking at the protein expression of HopM1 across these different temperatures. Figure 7 shows the results obtained from the western blot. We begin to slowly see production of HopM1 at 30°C beginning at hour 1 with maximal expression at hour 3 and HopM1 is slowly produced at 37°C beginning at hour 1 with maximal expression at hour 24. However, on closer inspection, we see that there are degradation products for HopM1 at 30°C and 37°C and that the bands appear a little lower than the expected size of 75kDa. These degradation products could possibly be the reason why HopM1 is able to grow on the

Sgal-ura-leu plates as seen with the serial dilution at 30°C and 37°C. Another possibility could be that the protein is unstable at these high temperatures. Literature also indicates that the effector proteins from *Pseudomonas syringae* pv. *tomato* do not elicit an effect in tomato plants at temperatures higher than 25°C but instead prefer cool and moist climates to be able to elicit an effect (Preston, 2000)

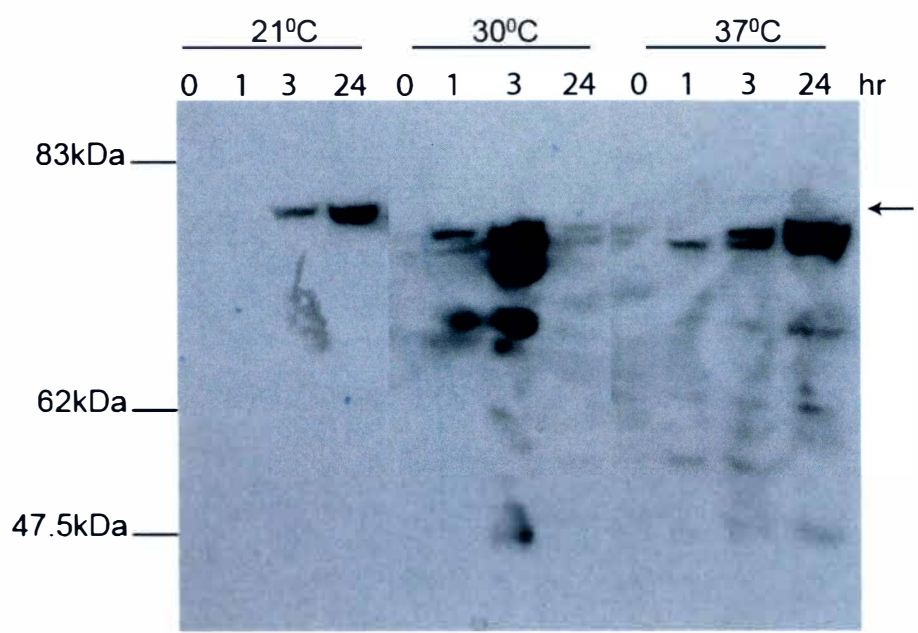


Figure 7. HopM1 is produced in yeast at all three temperatures. Yeast strain JGY4 that was co-transformed with plasmid pJB2 and pJB3 was induced with galactose for 48 hours. Aliquots of the strain were taken at respective time points and the extracts were prepared.

Localization of HopM1

We next wanted to look at the cellular localization of HopM1. Strains containing the control plasmid and HopM1 were induced with galactose and prepared for indirect immunofluorescence. At 3 hours after induction, we observed that HopM1 localized to punctate like structures in the cytoplasm of the cell as shown in Figure 8. There was no difference in observation at hour 24, which was when the maximum expression of HopM1 was observed in western blot analysis, or even at hour 48 after induction.

We utilized the stain Mitotracker Red (Invitrogen) to observe the localization of mitochondria in our strain expressing HopM1. Figure 8 shows the mitochondria stained as red thin tubules in the cell. Overlap images (Figure 9) were prepared to help determine if there was any co-localization between HopM1 and mitochondria. On observing the structures closely, we conclude that there is some overlap between the punctate structures that HopM1 localizes to and the mitochondria.

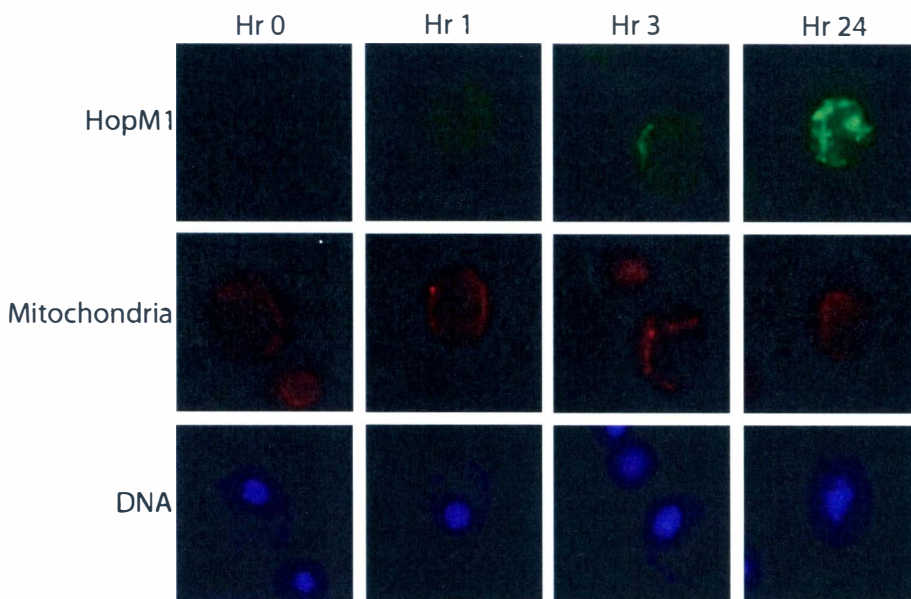


Figure 8. Localization of HopM1 in yeast. Yeast strain JGY4 containing HopM1 (pJB3) or control plasmid (pJG485) was grown in selective media containing 2% raffinose. HopM1 was induced after time point zero with the addition of 2% galactose. Aliquots were taken at each time point and fixed with 3.7% formaldehyde for 2 hours. Immunofluorescence was used to visualize the V5 epitope, Mitotracker Red and DAPI to visualize mitochondria and dna respectively.

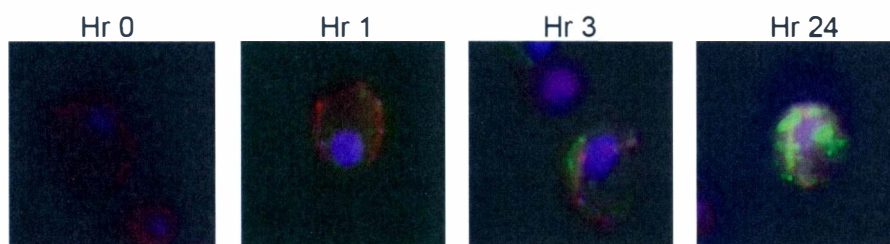


Figure 9. Overlay of yeast expressing HopM1. Yeast strain JGY4 containing HopM1 (pJB3) was grown in selective media containing 2% raffinose. HopM1 was induced after time point zero with the addition of 2% galactose. Aliquots were taken at each time point and fixed with 3.7% formaldehyde for 2 hours. Immunofluorescence was used to visualize the V5 epitope, Mitotracker Red and DAPI staining to visualize the mitochondria and dna respectively.

Cellular Fractionation of Yeast Strains

We moved to performing cellular fractionation and sucrose density gradient centrifugation of yeast strains containing the HopM1 or control plasmids to further determine HopM1 localization. Fractions resulting from the sucrose density gradient centrifugation were separated using an 8% SDS polyacrylamide gel. The resulting gel was transferred to a PVDF membrane and probed with the antibody for the V5 epitope. Figures 11 and 12 show the results of the western blots for the yeast strain containing the HopM1 plasmids and control plasmids. On looking at Figure 11, we see that HopM1 (75kDa) appears in the lanes indicated as P1, S1, P2, S2, P3 and P2t. In each of these fractions, we expect to see the presence of HopM1 (75kDa) as these fractions contain a combination of plasma membrane vesicles, mitochondria and secretory vesicles. The bands that appear towards the bottom of each lane are indication of non-specific binding and this is confirmed by the results as shown in figure 12 that shows the western blot analysis of the yeast strain containing the control vector. Figure 10 shows a schematic diagram of the cellular fractionations and sucrose density gradient centrifugation protocol.

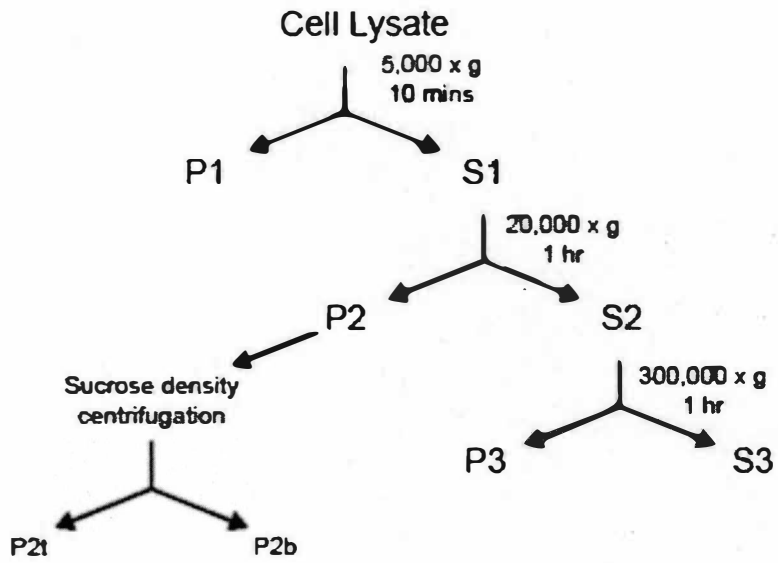


Figure 10. Diagram of cell fractionation and sucrose density centrifugation protocol.

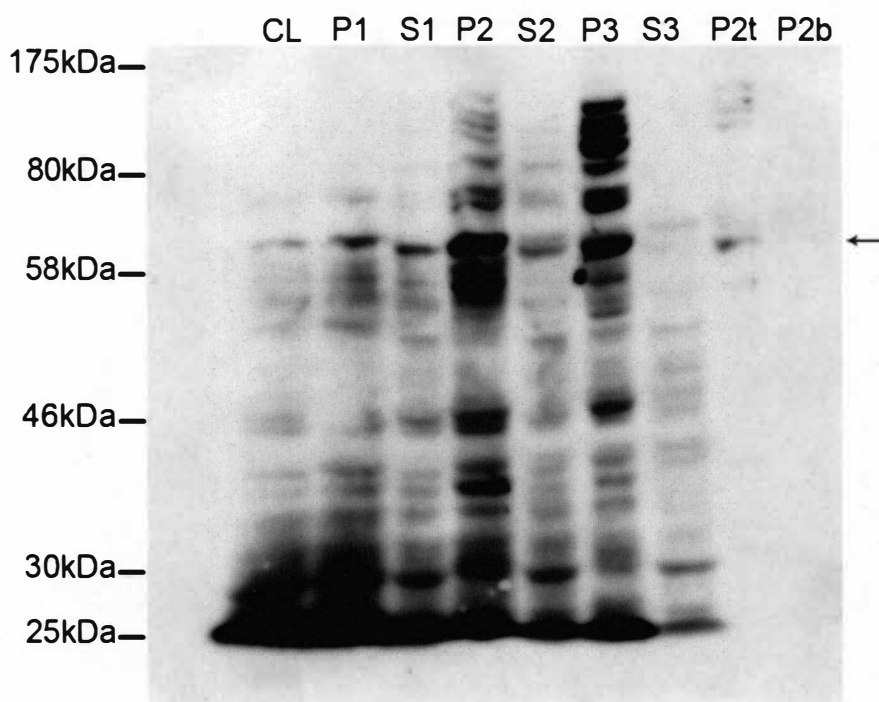


Figure 11. Western Blot Analysis of cellular fractionation and sucrose gradient density centrifugation of yeast strain containing pJB2 and pJB3. The levels of HopM1-V5 in the fractions resulting from the cellular fractionation (labeled CL, P1, S1, P2, S2, P3 and S3) and fractions from sucrose gradient density centrifugation (labeled P2t and p2b) were determined by immunoblotting. Molecular weight marker is shown. Arrow indicates the expected molecular weight (75kDa) of HopM1 protein.

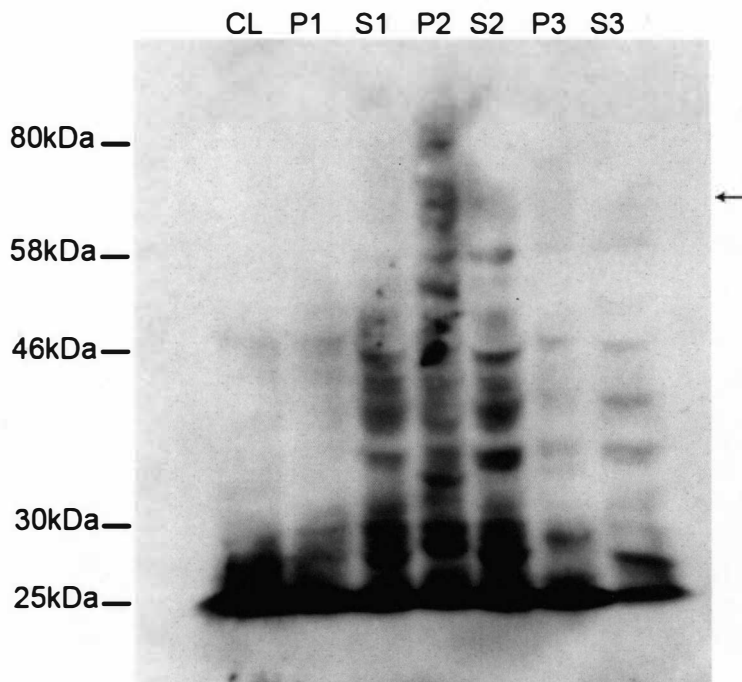


Figure 12. Western Blot analysis of cellular fractionation and sucrose density gradient centrifugation of yeast strain containing control plasmids. The levels of HopM1-V5 in the fractions resulting from the cellular fractionation (labeled CL, P1, S1, P2, S2, P3 and S3) were determined by immunoblotting. Molecular weight marker is shown. Arrow indicates the expected molecular weight (75kDa) of HopM1 protein.

Effect of HopM1 is Not Affected in a Yeast Strain Lacking the Mitochondria

In order to determine if the expression of a petite yeast strain containing either HopM1 or the empty vector changed, we performed serial dilution replica plating of the petite yeast strains before inducing with 2% galactose

and 24 hours after induction onto YPD, Sd-ura-leu, Sgal-ura-leu, Sglycerol-ura-leu and Sglycerol-ura-leu supplemented with galactose, and allowed the plates to incubate at 21°C for 1 week. We also performed serial dilution replica plating of the wild type yeast strain (JGY4) containing HopM1 or the empty vector under the same conditions onto YPD, Sd-ura-leu, Sgal-ura-leu, Sglycerol-ura-leu and Sglycerol-ura-leu supplemented with galactose.

Figure 13 and 14 show the results of the replica plating. The petite yeast strain (yeast strain lacking mitochondria – see figure 13) containing the HopM1 plasmids and the control plasmids were not able to grow on the media containing glycerol. This is due to the fact that yeast strains lacking mitochondria are not able to grow on respiratory media such as glycerol. Figure 14 shows the results for the wild type yeast strain (JGY4). We are able to see growth on the media containing glycerol. From both these results, we are able to determine that despite removing the mitochondria, we are still able to see the lethal effects of HopM1.

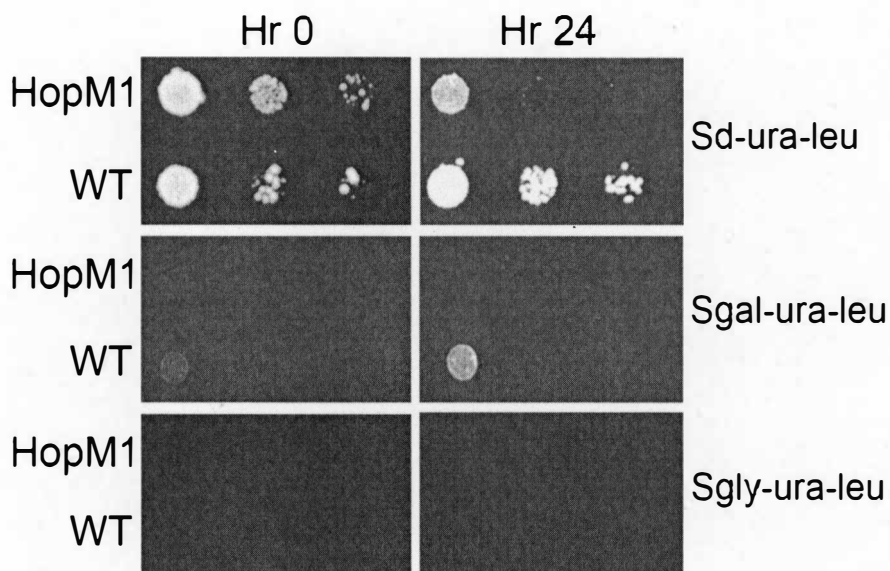


Figure 13. Effects of HopM1 on yeast strain lacking mitochondria. Yeast lacking mitochondria show a lethal phenotype. Petite yeast strain containing control (pJG484 and pJG485) and HopM1 (pJB2 and pJB3) plasmids were replica plated onto SD (SD-ura-leu), Sgal (Sgal-ura-leu) and Sglycerol (Sgly-ura-leu) medium and incubated at 21°C for 4 days. Each spot of cells is a 40-fold dilution of cells in the previous spot.

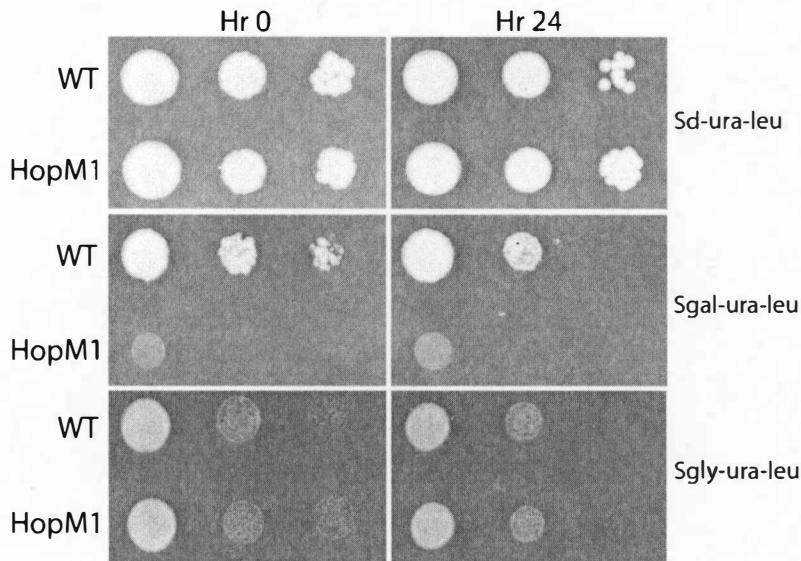


Figure 14. Mitochondria are still functional in yeast strain expressing HopM1. Yeast strain (JGY4) containing control (pJG484 and pJG485) and HopM1 (pJB2 and pJB3) plasmids were replica plated onto SD (SD-ura-leu), Sgal (Sgal-ura-leu) and Sglycerol (Sgly-ura-leu) medium and incubated at 21°C for 4 days. Each spot of cells is a 40-fold dilution of cells in the previous spot.

Discussion

We were able to successfully create a *Pseudomonas syringae* effector model system in *Saccharomyces cerevisiae* to study the effects of the effector proteins from *Pseudomonas syringae* pv. *tomato* DC3000. In order to be able to determine if HopM1 showed a phenotype in yeast, we

created the yeast plasmids pJB2 and pJB3, which contain *hopM1* under the control of *GAL1* with either the *LEU2* or *URA3* autotrophic marker for selection. Plasmids were transformed into yeast strain JGY4 and selected on media lacking uracil and leucine to isolate the yeast strain containing both plasmids. Western blot analysis was performed to show that our model was in fact working as expected. Results from the Western blot show that HopM1 expression begins at hour 3 with maximal expression at hour 24, at the expected molecular weight of 75kDa.

We have been able to show that HopM1 is able to inhibit the growth of yeast on solid media at 21°C. These results contradict the results that were published by Munkvold et al., (2008), which states that HopM1 is not lethal to the yeast on solid media at 30°C. The discrepancy in these results are due to the fact that we examined the effect HopM1 had on yeast at numerous temperatures and found that at 21°C, HopM1 was lethal to the yeast. On looking at the expression of HopM1 at 30°C and 37°C, we see that HopM1 is expressed at both these temperatures but also observe degradation bands at these temperatures. These degradation bands could point to the reason behind why HopM1 is able to grow on Sgal-ura-leu plates as seen with the serial dilution at 30°C and 37°C. Another reason could

be the protein is unstable at these higher temperatures. According to literature, the bacterium *Pseudomonas syringae* pv. *tomato* DC3000 elicits an effect in its host plants at cool moist temperatures between 18°C and 25°C (Preston, 2000) and at temperatures above this; only a minimal effect if any is seen. This is what led us to choosing 21°C as one of our parameters.

Next, we wanted to determine if HopM1 had a cytotoxic or cytostatic effect and this was done by looking at the number of viable cells after removal from the inducer. Our results showed that HopM1 does not have an outright cytotoxic effect unlike YopO (Nejedlik et al., 2004). Instead, HopM1 proved to be unique. Even at the time point that corresponds to the maximal expression of HopM1 (from western blot analysis), there were about 91% viable cells and at hour 48, there were about 37.5% viable cells. This hints at the possibility that HopM1 has a cytostatic effect; where the cells have slowed down in cycling through the growth cycle, or they have stopped dividing altogether but are able to grow once removed from the presence of the inducer.

Upon looking at the localization of HopM1, we were able to observe HopM1 localizing to punctate structures in the cytoplasm of the cell, with no obvious localization to

the nucleus as shown by DAPI staining beginning at hour 3 after induction. We utilized the stain Mitotracker Red to help determine if HopM1 was localizing to the mitochondria. We observed that HopM1 not only localized to the mitochondria but to punctate structures in the cytoplasm. In yeast, organelles such as the mitochondria, endoplasmic reticulum, golgi apparatus, endosomes and vesicles, all membrane bound organelles, appear as punctate structures when viewed under microscopy. Actin localization was also examined and we saw no obvious actin disruption (results not shown). We moved to performing cellular fractionation and separation of fractions on sucrose density gradients to determine the identity of these organelles.

Results from the western blot analysis of the fractions from cellular fractionations and sucrose density gradients all point to HopM1 co-sedimenting to the mitochondria and organelles or vesicles of the secretory pathway; as indicated by the presence of HopM1 in the fractions containing mitochondria, plasma membranes, endoplasmic reticulum and golgi.

The result that we obtained from HopM1 localization is similar to results obtained by Nomura et al., 2006. They looked at the expression of HopM1 in *Arabidopsis* cell fractions of the total membrane, soluble fraction, plasma

membrane and endomembrane and discovered that HopM1 is found in the endomembrane fraction and not the rest. This would be consistent with the fact that the endomembrane system is comprised of the endoplasmic reticulum, golgi apparatus, lysosomes, vacuoles, vesicles and cell membranes.

We obtained interesting results when trying to determine if the expression of HopM1 varied in the different strains of yeast. When expressed in the haploid yeast strain JGY4, HopM1 produced a lethal effect, and this is shown with the yeast strain containing HopM1 not being able to grow on Sgal-ura-leu plates. When HopM1 was expressed in the diploid strain of yeast (JGY709), the cells were able to grow on both Sd-ura-leu and Sgal-ura-leu plates. This was surprising, as we had expected that the diploid strain would have also produced the lethal effect as seen in the haploid strain.

To further understand if HopM1 localization was affected by mitochondria, we proceeded to create a petite yeast strain (rho-zero) that lacked mitochondrial DNA. Rho-zero yeast strains are able to grow slowly on media containing glucose but are not able to grow on respiratory media such as media glycerol or ethanol. From looking at the results presented, we are able to conclude that the

effects of HopM1 are not affected by the absence of mitochondria. As presented in an earlier section, we have shown that HopM1 localizes to the mitochondria (see figure 8, 9 and 11) but binding to the mitochondria alone is not sufficient for the lethal effects of HopM1 as observed from figure 13. Rather, HopM1 is affecting the mitochondria and the secretory pathway to cause its effect in yeast.

CHAPTER 3

ISOLATION OF SPONTANEOUS SUPPRESSORS OF HOPM1 IMPOSED LETHALITY

Introduction

Spontaneous suppressors are an invaluable tool for elucidating the role/identity of unknown cellular targets (Prelich G, 1999). Since first being described by Sturtevant in 1920 when looking at gynandromorphism and the exception displayed by vermilion eye color in *Drosophilla melanogaster* (Sturtevant, A.H., 1920), suppressors have been used to study genetic pathways (Prelich G, 1999).

As presented in an earlier chapter (see chapter 2), HopM1 has a lethal phenotype when expressed in yeast. The goal with creating spontaneous suppressors is to be able to identify cellular targets of HopM1 and the gene(s) and their protein products that are required for the imposed lethality. Using this screen, we identified 19 spontaneous revertants of HopM1 lethal phenotype. We were also able to further characterize these suppressors by growth rates, protein expression levels of HopM1, localization and ability to sporulate.

Materials and Methods

Media

Media for yeast growth such as YPD, SD-ura, SD-ura-leu, Sgal-ura, and Sgal-ura-leu have been previously described (Guthrie and Fink, 1991). Sporulation media was made as described by Guthrie and Fink, 1991.

Lithium Acetate Yeast Transformation

Yeast strains were transformed as described (Gietz and Schiestl, 1991).

Creation of Suppressor Strains

Plasmids pJB2 (HopM1 *LEU2*) and pJB3 (HopM1 *URA3*) were co-transformed into haploid yeast strain JGY4 (see table 3). Two plasmids were used in order to determine that the mutation we generated and isolated occurred in the yeast genome and not in the plasmids. If for example the mutation were to occur in the *GAL1* promoter of one plasmid resulting in no expression of HopM1, the other plasmid would still function normally and allow us to proceed with our designed screen. Independent transformants that were isolated were grown overnight in Sraffinose-ura-leu media, with raffinose being used as the sole carbon source to help prime the yeast and prepare them for transitioning into galactose.

The overnight cultures were induced with galactose to turn on expression of HopM1 and 24 hours following induction, 10^7 cells were plated onto Sgal-ura-leu. The plates were allowed to grow at 21°C for 5 days.

Sporulation

Suppressor strains were grown in 5ml of sporulation media with rotation at room temperature for 5 days after which 100 μ l samples were collected from each strain and digested with 3 μ l Zymolyase 20T for 10-20 minutes. Treatment with Zymolyase allows for the weakening of the spore ascus by the digestion of β 1-3 glucanase, thus enabling ease of separation of individual spores. 20 μ l of the digested sample is then plated down the centre of a YPD plate and dissected under a Nikon Eclipse E400 Microscope using a micromanipulator. Dissection was carried out as described by Guthrie and Fink, 1991. The dissected plates were allowed to sit at room temperature for 4 days to allow for growth of spores, after which both HopM1 plasmids were transformed back in and transformants were screened to look for the ability to survive HopM1 imposed lethality. Once the spores containing the suppressor gene were identified, they were mated with yeast strain JGY3 that is of mating

type a (See Table 3), and the whole process was repeated to obtain heterozygous diploid suppressors.

Strain	Chromosome
JGY3	MATa ade2-101 his3-200 leu2-3, 112 ura3-52
JGY4	MAT α lys2-801 his3-200 leu2-3, 112 ura3-52

Table 3. Yeast strains used.

Titer Assay

A titer assay of all haploid suppressors was performed as previously described in chapter 2.

Western Blotting

Western Blotting of all haploid suppressors was performed as previously described in chapter 2.

Hop Immunofluorescence

Immunofluorescence was performed as previously described in chapter 2.

High Molecular Weight Yeast DNA

High Molecular Weight Yeast DNA was prepared as described by Guthrie and Fink, 1991 with modifications. Essentially, yeast strains were grown for 2 days in a liter of YPD media at 21°C. Once cells had grown to 2×10^8 cells/ml,

the cultures were harvested and centrifuged in a Sorvall RC 5B Plus centrifuge using a SS-34 rotor at 4°C for 10 minutes at 500g. The resulting pellet was then washed once with 1/5th volume of ice-cold 50mM EDTA and resuspended in 10ml of Tris-HCl pH8.0 with 2% v/v 2-mercaptoethanol and allowed to incubate for 15 minutes at room temperature. The resuspended cells were then centrifuged again. The supernatant was poured off and the pellet was resuspended in the remaining supernatant and layered over liquid nitrogen drop by drop in a cooled pestle and mortar. The droplets are then ground up in the pestle and mortar until it forms a white powder. Liquid nitrogen is added as needed to keep powder cold. Once this is accomplished, the powder is resuspended in 1/200th volume of Lysis Buffer (0.1M Tris-HCl pH8.0, 0.1M EDTA, 0.15M NaCl and 2% 2 mercaptoethanol) to form spheroplasts. The spheroplasts are lysed further by 3 fold dilution into Lysis Buffer made 4% v/v with Sarkosyl and incubated at 37°C for 20 minutes. Following the 20 minute incubation, equal volume of Lysis Buffer (0.1M Tris-HCl pH8.0 and 4% Sarkosyl) is added and incubated at 70°C for 15 minutes. DNase-free RNase (0.1mg/ml) is added to the culture and allowed to incubate at 37°C for 1 hour after which Proteinase K (Sigma) is added to a final concentration of 1.33mg/ml in two aliquots at hourly

intervals and allowed to continue incubating at 37°C for a total of 2 hours. After the incubation, the cells are incubated at 70°C for 15 minutes. Phenol/Chloroform/Isoamyl alcohol 25:25:1 v/v is added and allowed to rock gently at room temperature until a white emulsion forms (20 minutes). The phases are then separated by centrifugation at 20,000g at room temperature for 10 minutes in a bench top Eppendorf 5810R Centrifuge. The aqueous supernatant is removed to a new tube and incubated at 45°C to remove all traces of chloroform. To further isolate the high molecular weight DNA, 10ml of the sample is layered on 24ml preparative step sucrose gradients. The gradients are centrifuged in a Beckman Ultracentrifuge Optima XL-100K using a swing bucket (Beckman SW 28) at 33,000g for 17 hours at 4°C. 1ml samples are collected from the top of the gradient using a wide bore pipette. Small aliquots of the samples are then analyzed on a 1% Agarose gel. Samples containing the correct band size are pooled and dialyzed against 0.15M NaCl, 10mM Tris-HCl pH 8.0 and 1mM EDTA.

Size Fractionation of Partially Digested Genomic DNA

Genomic DNA that was obtained after dialyzing was partially digested as described by Maniatis et al., 1982. Briefly, aliquots of DNA were digested using a 10-fold

dilution of the restriction enzyme *Sau3A* to determine the ideal enzyme concentration that would yield maximum results. The ideal enzyme concentration was determined to be 1:5000 dilution per 10ng of DNA. The reaction was then scaled up to obtain a good yield of partially digested genomic DNA.

Dephosphorylation of Vector

The vector pRS313 (see table 4) was dephosphorylated as described in Guthrie and Fink, 1991. Shrimp Alkaline Phosphatase was used to remove the 5' Phosphates from the vector DNA.

Plasmid	Yeast	Bacteria
pRS313	<i>CEN6 ARSH4 HIS3</i>	f1 bla

Table 4. Plasmid used for cloning.

Results

Creation of Suppressors

Yeast strain JGY4 (Table 3) was transformed with two HopM1 expressing plasmids (pJB2 and pJB3). Plasmid pJB2 and pJB3 contain the *LEU2* and *URA3* auxotrophic marker

respectively. We began our screen with 30 independent populations. 30 different populations were started from 30 different colonies in different tubes. The yeast strain containing both plasmids were plated onto Sgal-ura-leu media at a concentration of 10^7 cells, which is the normal mutation rate of the auxotrophic marker Uracil (Geiser et al., 1993), and allowed to grow at room temperature for 5 days. Under normal conditions, when the yeast strain containing both HopM1 plasmids are plated onto Sgal-ura-leu media, growth of yeast strains on this media would be inhibited due to the lethality of HopM1.

Twenty of the plates produced colonies and the remaining ten were discarded. Three colonies from each of the 20 plates were selected for further characterization. Each potential suppressor was assayed to determine if it was able to lose both plasmids. This step was crucial to make sure that neither plasmid had integrated into the genome. Thirty of the sixty potential suppressor strains were able to lose both plasmids. Plasmids pJB2 and pJB3 were transformed back in to the 30 potential suppressors and allowed to grow at room temperature for 5 days. All potential suppressor strains were assayed for ability to grow when HopM1 was expressed from the *GAL1* promoter. Nineteen suppressor strains were obtained based on their

robust growth on Sgal-ura-leu media. The 19 strains contain 14 that are independent and 5 that may be siblings. These suppressors were named SupM1 1-19. See table 5.

Suppressor	Original Name	Ability to Sporulate	Western Blot Degradation
SupM1-1	1B2	No	Yes
SupM1-2	3A1	Yes	No
SupM1-3	3A2	Yes	No
SupM1-4	7A2	Yes	Yes
SupM1-5	8B1	Yes	No
SupM1-6	13A1	Yes	No
SupM1-7	13A2	Yes	No
SupM1-8	14A2	Yes	Yes
SupM1-9	16A1	Yes	No
SupM1-10	19A1	Yes	No
SupM1-11	19A2	No	Yes
SupM1-12	20A1	Yes	Yes
SupM1-13	21B1	Yes	No
SupM1-14	24A1	Yes	No
SupM1-15	24B1	Yes	No
SupM1-16	24B2	Yes	No
SupM1-17	25B1	Yes	No
SupM1-18	27B1	No	No
SupM1-19	29A	Yes	No

Table 5. Table of spontaneous suppressors, ability to sporulate and degradation pattern.

Expression of HopM1 in Some Haploid Suppressors Show Degradation of the Protein

HopM1 is unstable at 30°C and 37°C resulting in degraded fragments of HopM1 in the cell (chapter 2). HopM1 is only stable at 21°C and only is lethal at 21°C (chapter 2). Each strain was examined by Western blot analysis to determine if the HopM1 in each strain was stable or degraded at 21°C.

The suppressor strains containing the plasmids pJB2 and pJB3 were grown to approximately mid-log phase in minimal selective media. Galactose was added to the media to induce the production of HopM1. Aliquots were taken at 0, 1, 3, 24 and 48 hours and whole cell protein extracts were prepared and separated using an 8% SDS polyacrylamide gel. The resulting gel was transferred to a PVDF membrane and probed with the antibody for V5, an engineered c-terminal epitope on HopM1.

Figures 15-24 show the results obtained from the western blot analysis of the suppressors. The expected molecular weight of HopM1 is 75kDa. As shown in figures 15-17, we observe that SupM1 1, 4, 8, 11, and 12 show some degradation of HopM1 even when grown at 21°C. While we cannot conclusively attribute this to why suppression is occurring, it is likely that there is not sufficient HopM1 present to cause lethality. Thus these suppressor strains

may only cause suppression by destabilizing HopM1 further. In figures 18-24 we observe that Sup 2, 3, 5-7, 9, 10, 13-17, and 19 show no degradation of the HopM1 protein. The maximal production of HopM1 in each of the suppressor strains can be observed in the respective figures. Table 5 shows the combined results of suppressors that are able to sporulate and the stability of HopM1 protein in each suppressor strain.

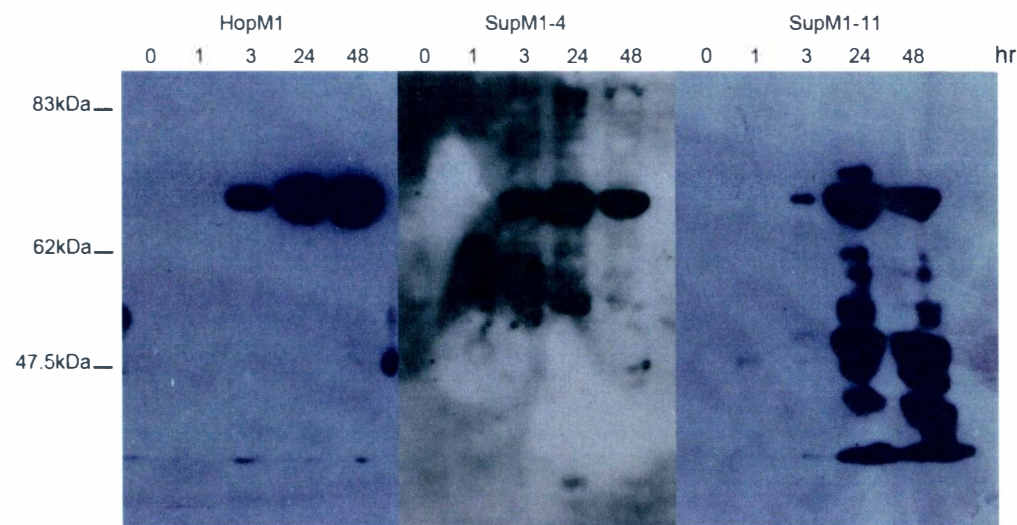


Figure 15. HopM1 is produced in WT and in suppressor SupM1-4 and SupM1-11. Yeast strain JGY4, SupM1-4 and SupM1-11 containing both HopM1 plasmids (pJB2 and pJB3) were induced with galactose at time zero, aliquots were taken and protein extracts prepared. The levels of HopM1-V5 in un-induced and induced cultures were determined by immunoblotting. Molecular weight marker is shown. HopM1 produces a band at the expected molecular weight of 75kDa, whereas SupM1-4 and SupM1-11 shows a degradation of the HopM1 protein.

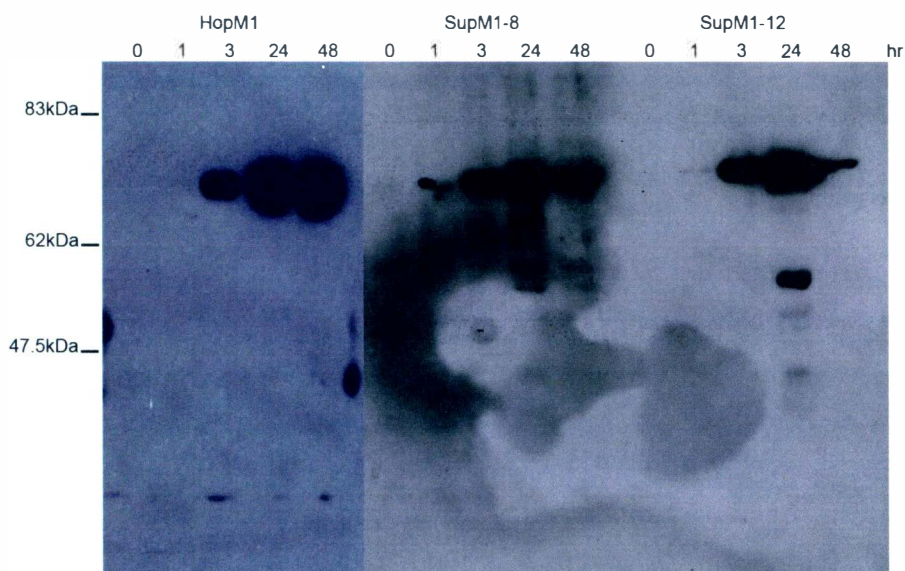


Figure 16. HopM1 is produced in WT and in suppressor SupM1-8 and SupM1-12. Yeast strain JGY4, SupM1-4 and SupM1-11 containing both HopM1 plasmids (pJB2 and pJB3) were induced with galactose at time zero, aliquots were taken and protein extracts prepared. The levels of HopM1-V5 in un-induced and induced cultures were determined by immunoblotting. Molecular weight marker is shown. HopM1 produces a band at the expected molecular weight of 75kDa, whereas SupM1-8 and SupM1-12 shows a degradation of the HopM1 protein.

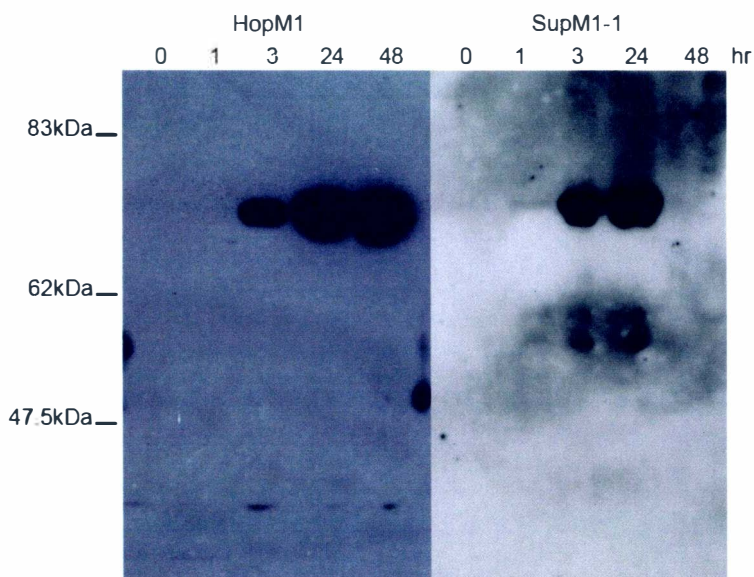


Figure 17. HopM1 is produced in WT and in suppressor SupM1-1. Yeast strain JGY4 and SupM1-1 containing both HopM1 plasmids (pJB2 and pJB3) were induced with galactose at time zero, aliquots were taken and protein extracts prepared. The levels of HopM1-V5 in un-induced and induced cultures were determined by immunoblotting. Molecular weight marker is shown. HopM1 produces a band at the expected molecular weight of 75kDa, whereas SupM1-1 shows a degradation of the HopM1 protein.

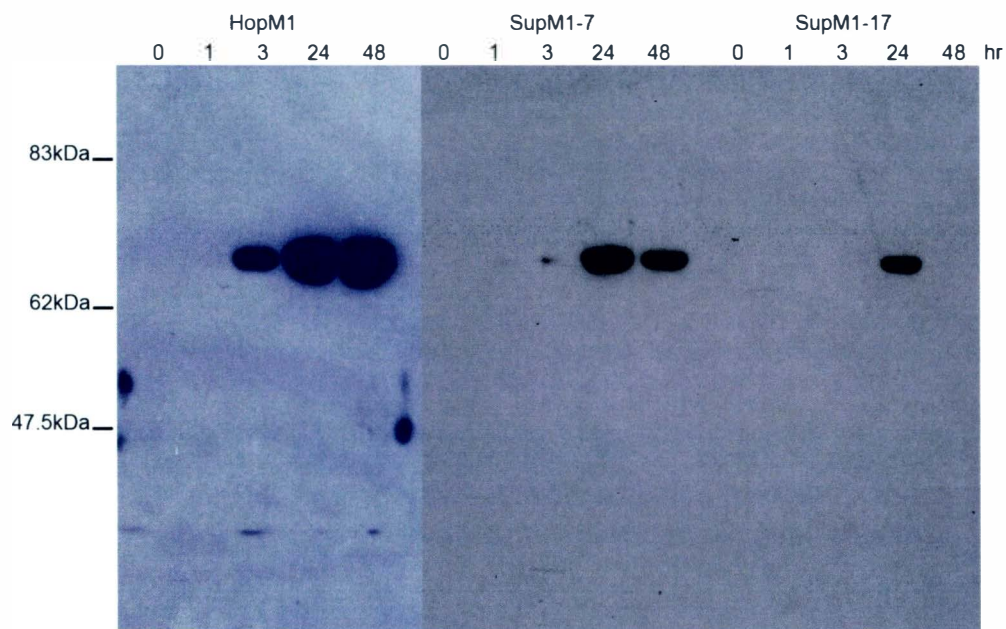


Figure 18. HopM1 is produced in WT and in suppressor SupM1-7 and SupM1-17. Yeast strain JGY4 and SupM1-7 and SupM1-17 containing both HopM1 plasmids were induced with galactose at time point zero, aliquots were taken and protein extracts prepared. The levels of HopM1-V5 in un-induced and induced cultures were determined by immunoblotting. Molecular weight marker is shown. HopM1, SupM1-7 and SupM1-17 produce a band at the expected molecular weight of 75kDa.

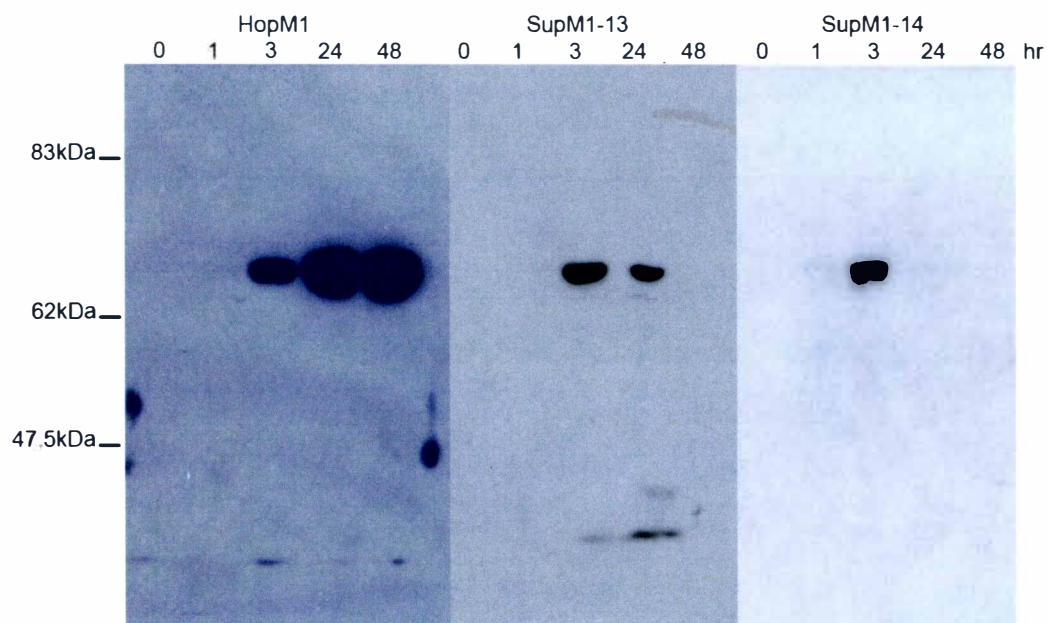


Figure 19. HopM1 is produced in WT and in suppressor SupM1-13 and SupM1-14. Yeast strain JGY4 and SupM1-13 and SupM1-14 containing both HopM1 plasmids were induced with galactose at time zero, aliquots were taken and protein extracts prepared. The levels of HopM1-V5 in un-induced and induced cultures were determined by immunoblotting. Molecular weight marker is shown. HopM1, SupM1-13 and SupM1-14 produce a band at the expected molecular weight of 75kDa.

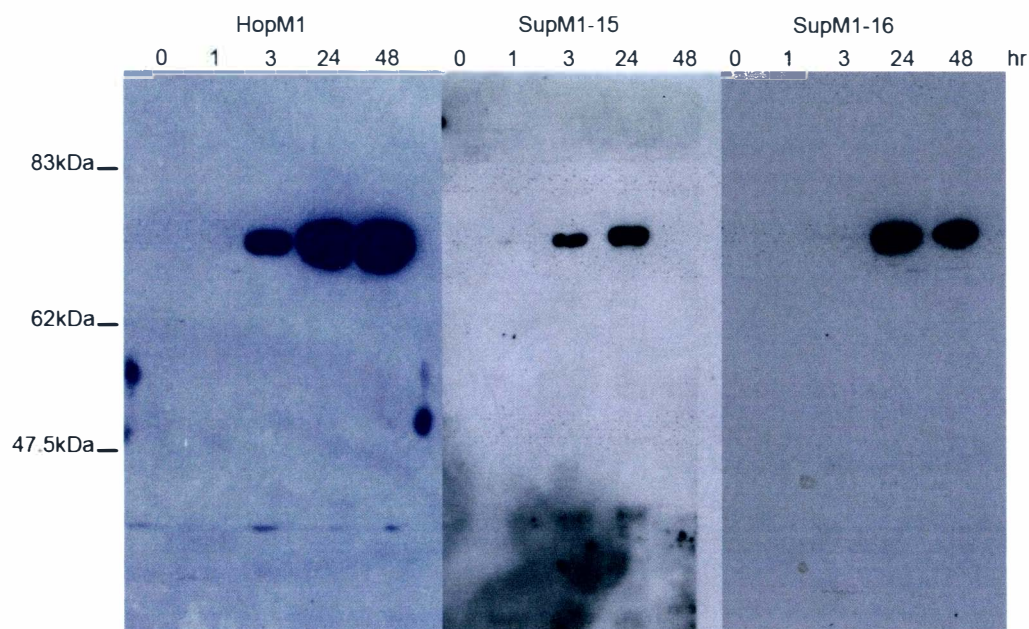


Figure 20. HopM1 is produced in WT and in suppressor SupM1-15 and SupM1-16. Yeast strain JGY4 and SupM1-15 and SupM1-16 containing both HopM1 plasmids were induced with galactose at time zero, aliquots were taken and protein extracts prepared. The levels of HopM1-V5 in un-induced and induced cultures were determined by immunoblotting. Molecular weight marker is shown. HopM1, SupM1-15 and SupM1-16 produce a band at the expected molecular weight of 75kDa.

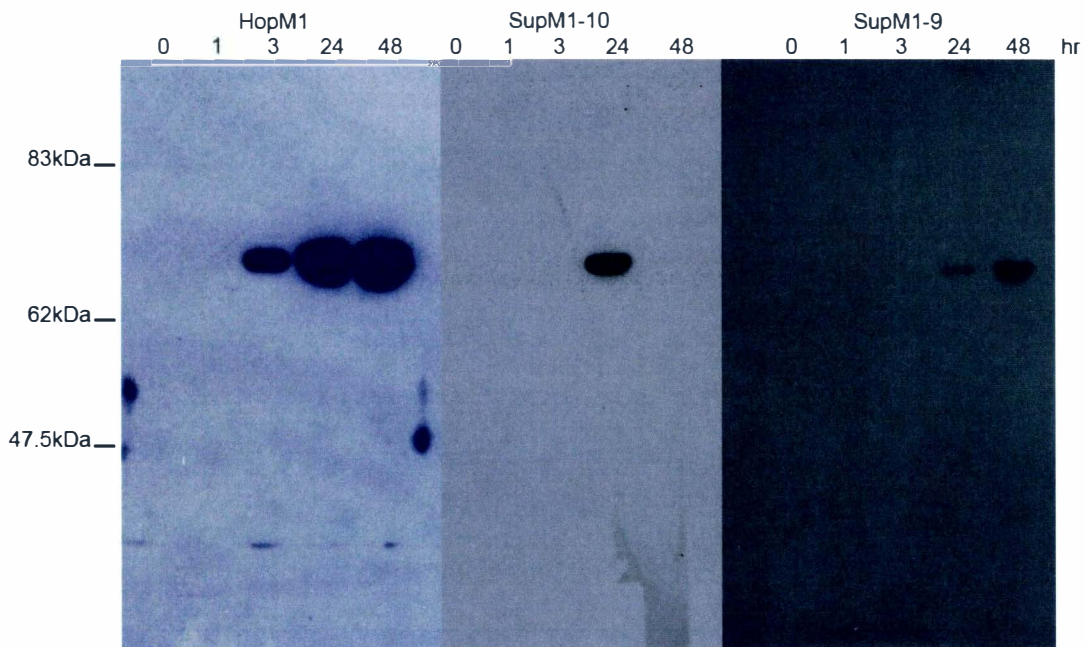


Figure 21. HopM1 is produced in WT and in suppressor SupM1-11 and SupM1-9. Yeast strain JGY4 and SupM1-10 and SupM1-9 containing both HopM1 plasmids were induced with galactose at time zero, aliquots were taken and protein extracts prepared. The levels of HopM1-V5 in un-induced and induced cultures were determined by immunoblotting. Molecular weight marker is shown. HopM1, SupM1-11 and SupM1-9 produce a band at the expected molecular weight of 75kDa.

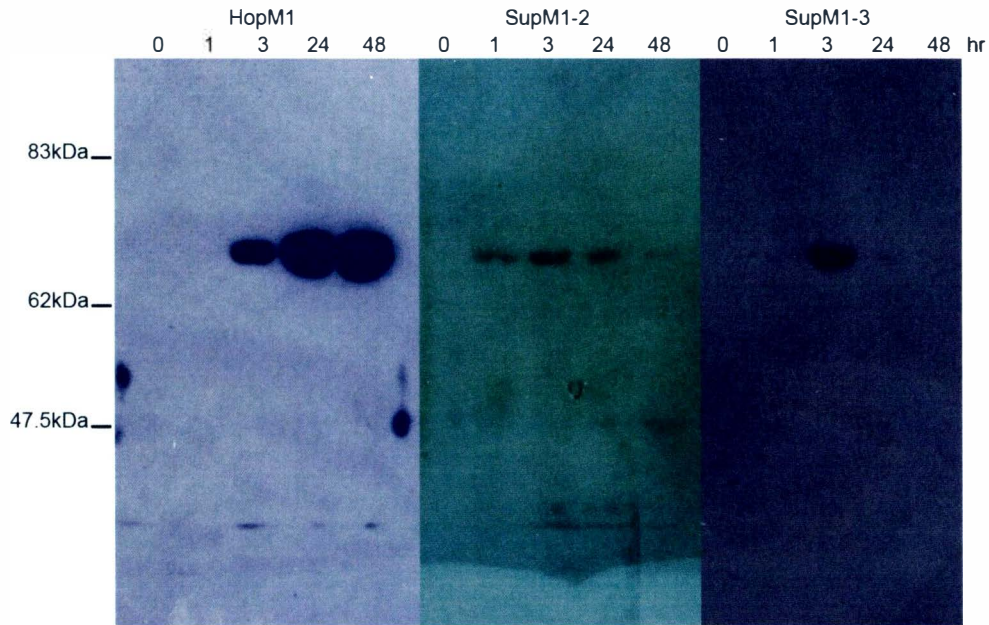


Figure 22. HopM1 is produced in WT and in suppressor SupM1-2 and SupM1-3. Yeast strain JGY4 and SupM1-2 and SupM1-3 containing both HopM1 plasmids were induced with galactose at time zero, aliquots were taken and protein extracts prepared. The levels of HopM1-V5 in un-induced and induced cultures were determined by immunoblotting. Molecular weight marker is shown. HopM1, SupM1-2 and SupM1-3 produce a band at the expected molecular weight of 75kDa.

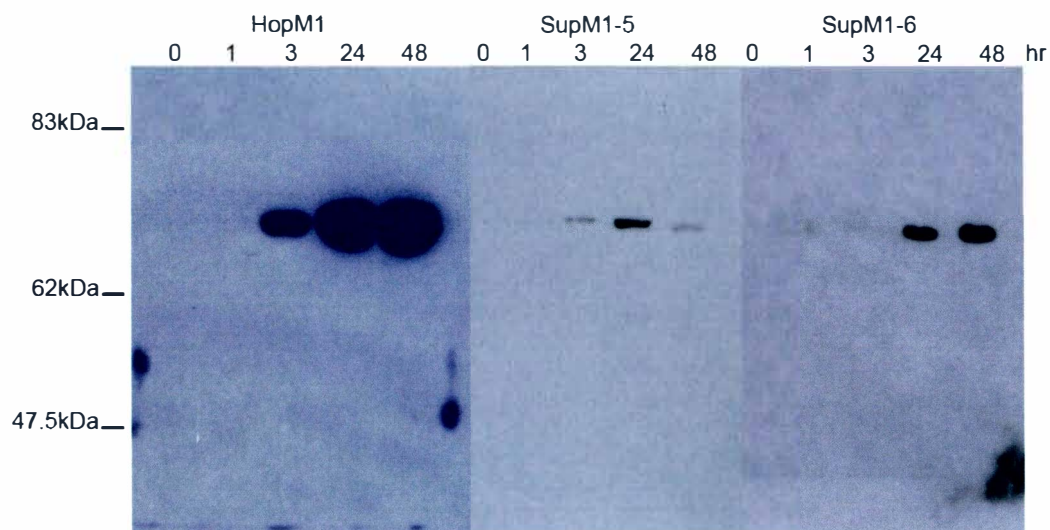


Figure 23. HopM1 is produced in WT and in suppressor SupM1-5 and SupM1-6. Yeast strain JGY4 and SupM1-5 and SupM1-6 containing both HopM1 plasmids were induced with galactose at time zero, aliquots were taken and protein extracts prepared. The levels of HopM1-V5 in un-induced and induced cultures were determined by immunoblotting. Molecular weight marker is shown. HopM1, SupM1-5 and SupM1-6 produce a band at the expected molecular weight of 75kDa.

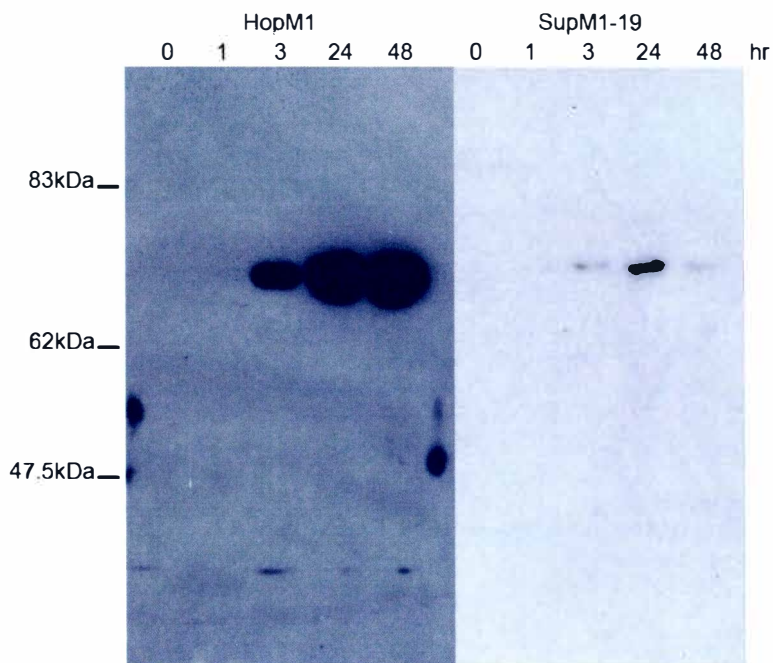


Figure 24. HopM1 is produced in WT and in suppressor SupM1-19. Yeast strain JGY4 and SupM1-19 containing both HopM1 plasmids were induced with galactose at time zero, aliquots were taken and protein extracts prepared. The levels of HopM1-V5 in un-induced and induced cultures were determined by immunoblotting. Molecular weight marker is shown. HopM1 and SupM1-19 produce a band at the expected molecular weight of 75kDa.

Localization of HopM1 in Suppressor Strains

As described in Chapter 2, HopM1 localizes to the organelles or vesicles of the secretory pathway. HopM1 appears in punctate like structures in the cytoplasm of wild type yeast expressing HopM1 with no obvious localization to the nucleus. The suppressor strains containing the HopM1 plasmid were induced with galactose and prepared for indirect immunofluorescence. We observed no difference in localization of HopM1 in any of our suppressor strains as shown in Figures 25-37. In each suppressor strain, HopM1 localized to punctate structures in the cytoplasm with no obvious localization to the nucleus, beginning at hour 1.

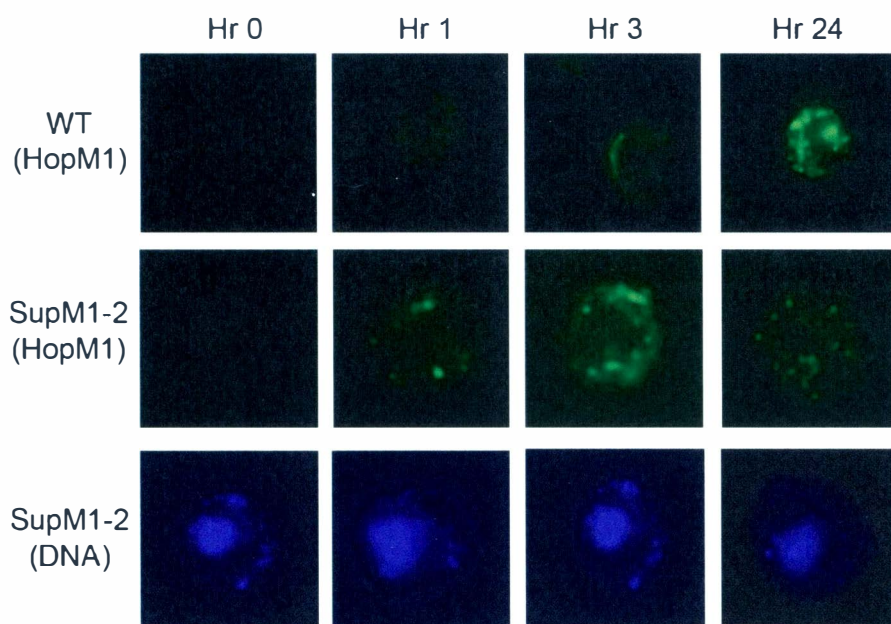


Figure 25. Localization of HopM1 in WT and suppressor yeast strain SupM1-2. Yeast strain JGY4 containing HopM1 (pJB3) and suppressor strain SupM1-2 were grown in selective media containing 2% raffinose. HopM1 was induced after time point zero with the addition of 2% galactose. Aliquots were taken at each time point and fixed with 3.7% formaldehyde for 2 hours. Immunofluorescence was used to visualize the V5 epitope and DAPI to visualize DNA respectively.

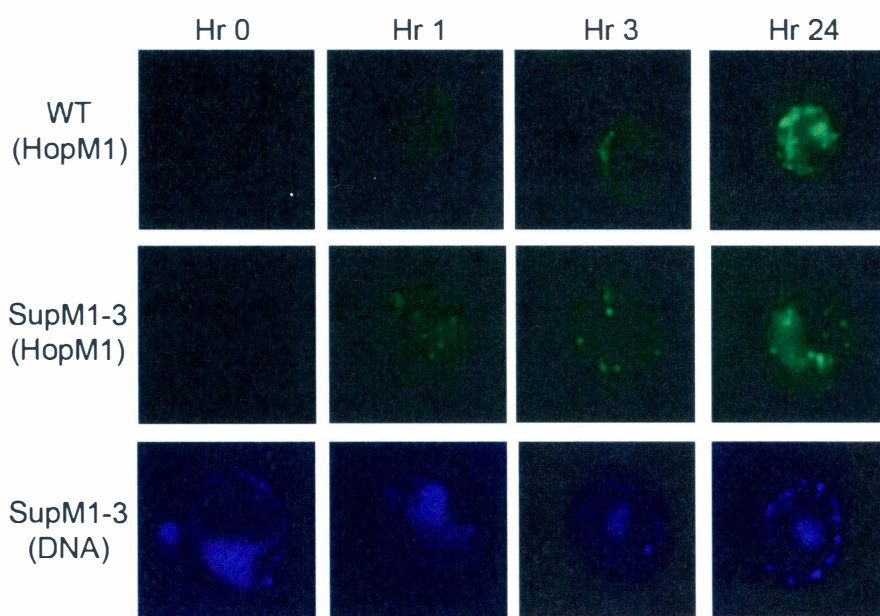


Figure 26. Localization of HopM1 in WT and suppressor yeast strain SupM1-3. Yeast strain JGY4 containing HopM1 (pJB3) and suppressor strain SupM1-3 were grown in selective media containing 2% raffinose. HopM1 was induced after time point zero with the addition of 2% galactose. Aliquots were taken at each time point and fixed with 3.7% formaldehyde for 2 hours. Immunofluorescence was used to visualize the V5 epitope and DAPI to visualize DNA respectively.

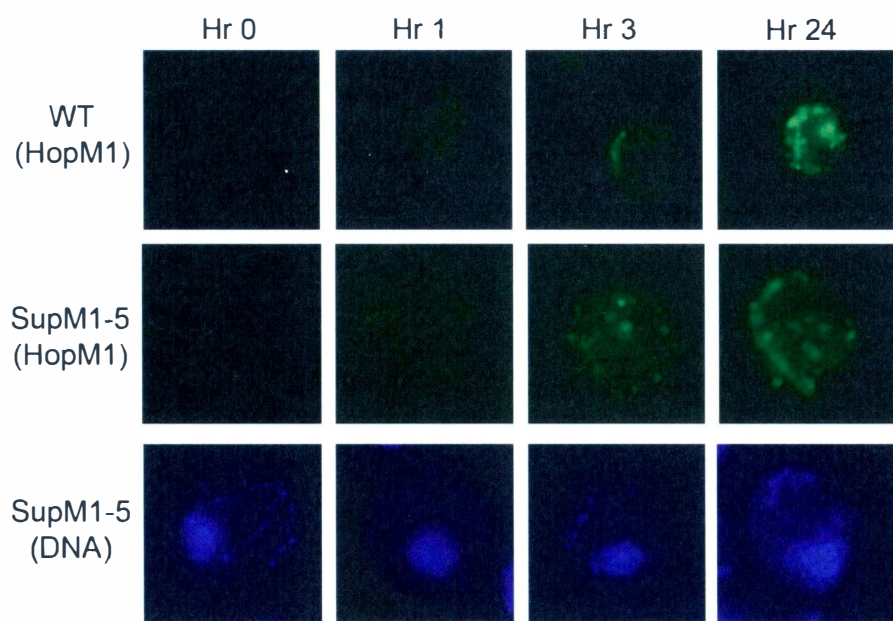


Figure 27. Localization of HopM1 in WT and suppressor yeast strain SupM1-5. Yeast strain JGY4 containing HopM1 (pJB3) and suppressor strain SupM1-5 were grown in selective media containing 2% raffinose. HopM1 was induced after time point zero with the addition of 2% galactose. Aliquots were taken at each time point and fixed with 3.7% formaldehyde for 2 hours. Immunofluorescence was used to visualize the V5 epitope and DAPI to visualize DNA respectively.

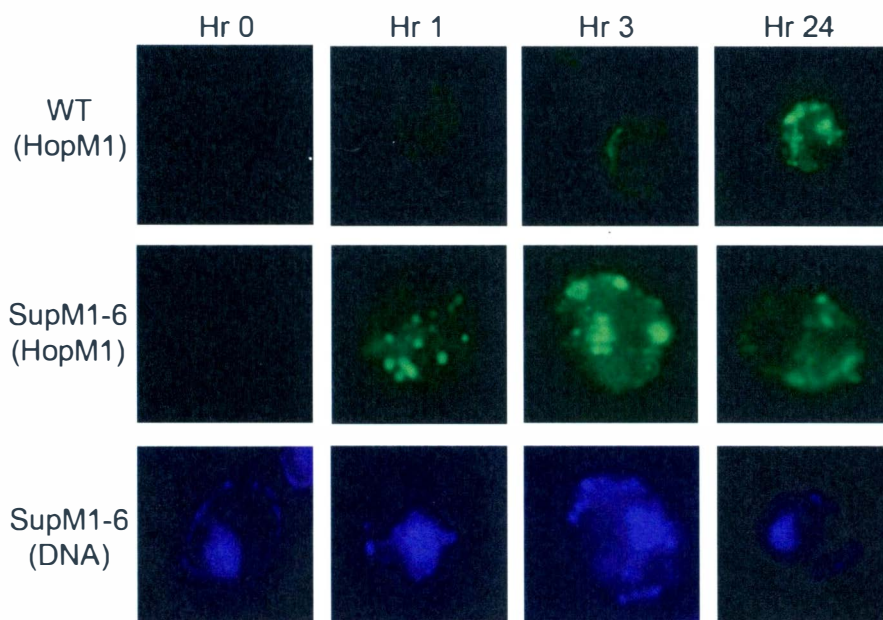


Figure 28. Localization of HopM1 in WT and suppressor yeast strain SupM1-6. Yeast strain JGY4 containing HopM1 (pJB3) and suppressor strain SupM1-6 were grown in selective media containing 2% raffinose. HopM1 was induced after time point zero with the addition of 2% galactose. Aliquots were taken at each time point and fixed with 3.7% formaldehyde for 2 hours. Immunofluorescence was used to visualize the V5 epitope and DAPI to visualize DNA respectively.

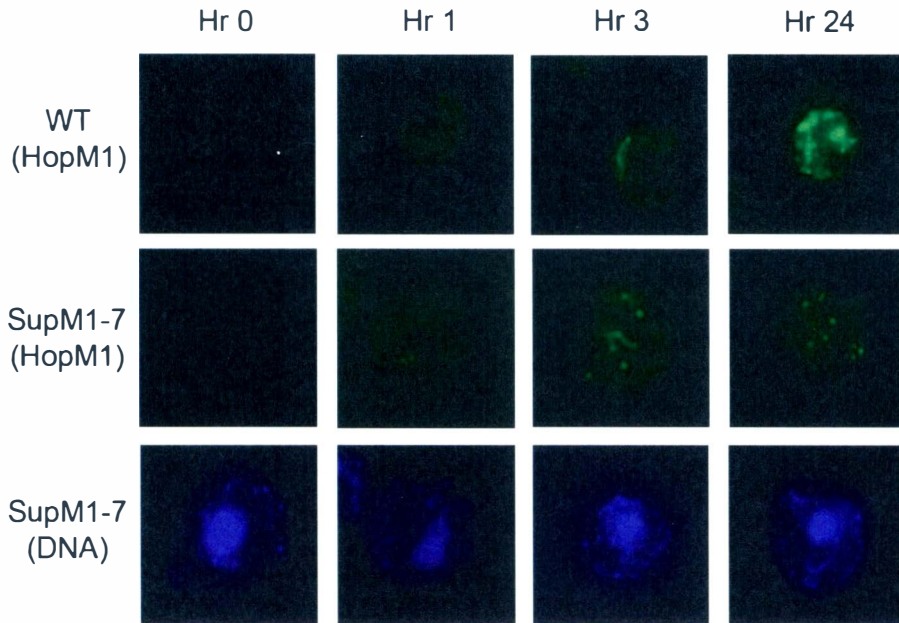


Figure 29. Localization of HopM1 in WT and suppressor yeast strain SupM1-7. Yeast strain JGY4 containing HopM1 (pJB3) and suppressor strain SupM1-7 were grown in selective media containing 2% raffinose. HopM1 was induced after time point zero with the addition of 2% galactose. Aliquots were taken at each time point and fixed with 3.7% formaldehyde for 2 hours. Immunofluorescence was used to visualize the V5 epitope and DAPI to visualize DNA respectively.

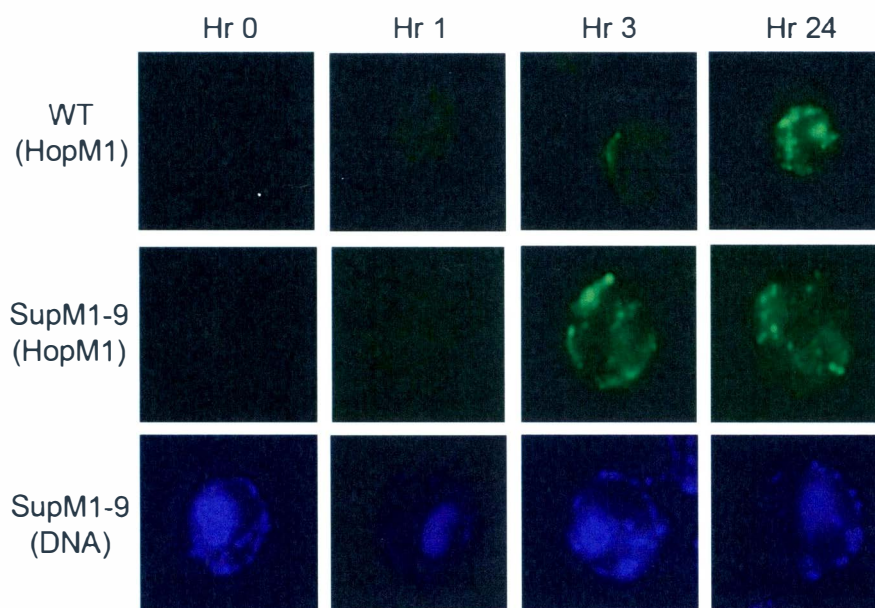


Figure 30. Localization of HopM1 in WT and suppressor yeast strain SupM1-9. Yeast strain JGY4 containing HopM1 (pJB3) and suppressor strain SupM1-9 were grown in selective media containing 2% raffinose. HopM1 was induced after time point zero with the addition of 2% galactose. Aliquots were taken at each time point and fixed with 3.7% formaldehyde for 2 hours. Immunofluorescence was used to visualize the V5 epitope and DAPI to visualize DNA respectively.

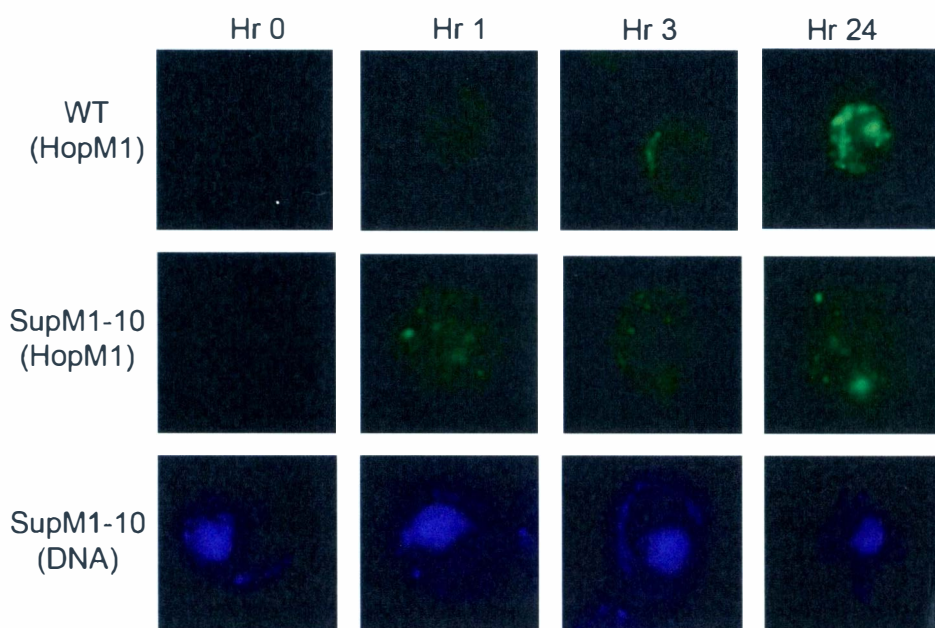


Figure 31. Localization of HopM1 in WT and suppressor yeast strain SupM1-10. Yeast strain JGY4 containing HopM1 (pJB3) and suppressor strain SupM1-10 were grown in selective media containing 2% raffinose. HopM1 was induced after time point zero with the addition of 2% galactose. Aliquots were taken at each time point and fixed with 3.7% formaldehyde for 2 hours. Immunofluorescence was used to visualize the V5 epitope and DAPI to visualize DNA respectively.

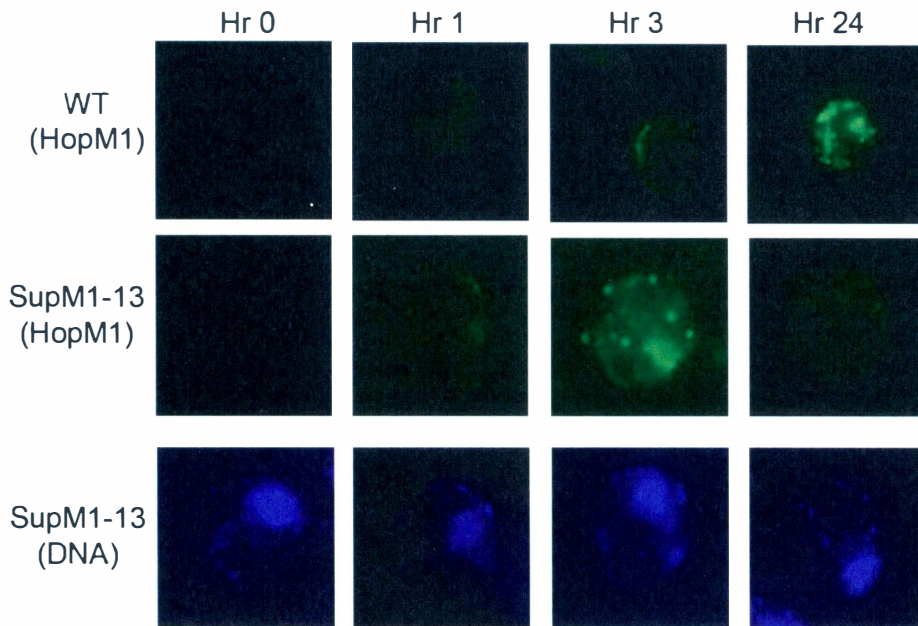


Figure 32. Localization of HopM1 in WT and suppressor yeast strain SupM1-13. Yeast strain JGY4 containing HopM1 (pJB3) and suppressor strain SupM1-13 were grown in selective media containing 2% raffinose. HopM1 was induced after time point zero with the addition of 2% galactose. Aliquots were taken at each time point and fixed with 3.7% formaldehyde for 2 hours. Immunofluorescence was used to visualize the V5 epitope and DAPI to visualize DNA respectively.

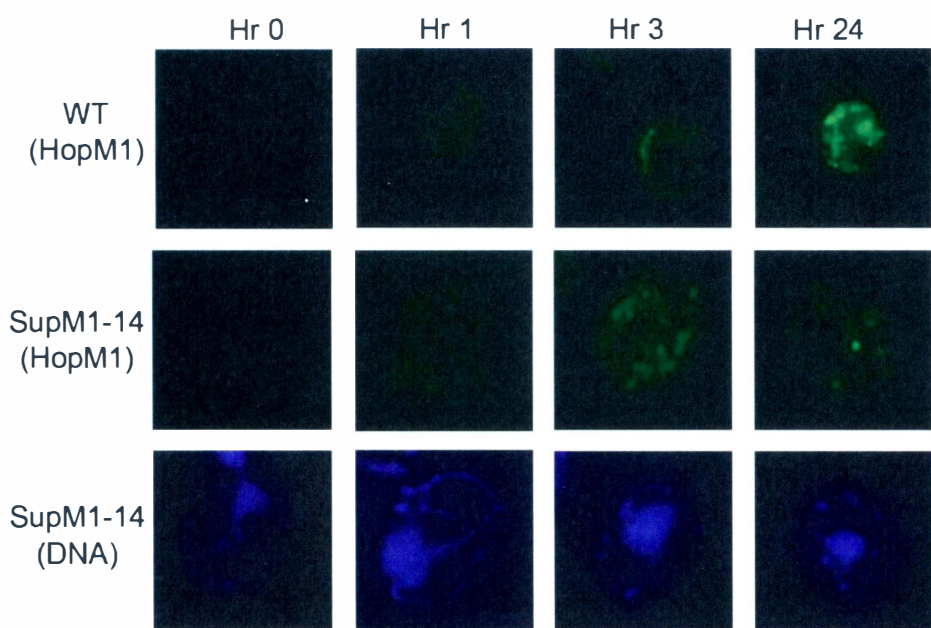


Figure 33. Localization of HopM1 in WT and suppressor yeast strain SupM1-14. Yeast strain JGY4 containing HopM1 (pJB3) and suppressor strain SupM1-14 were grown in selective media containing 2% raffinose. HopM1 was induced after time point zero with the addition of 2% galactose. Aliquots were taken at each time point and fixed with 3.7% formaldehyde for 2 hours. Immunofluorescence was used to visualize the V5 epitope and DAPI to visualize DNA respectively.

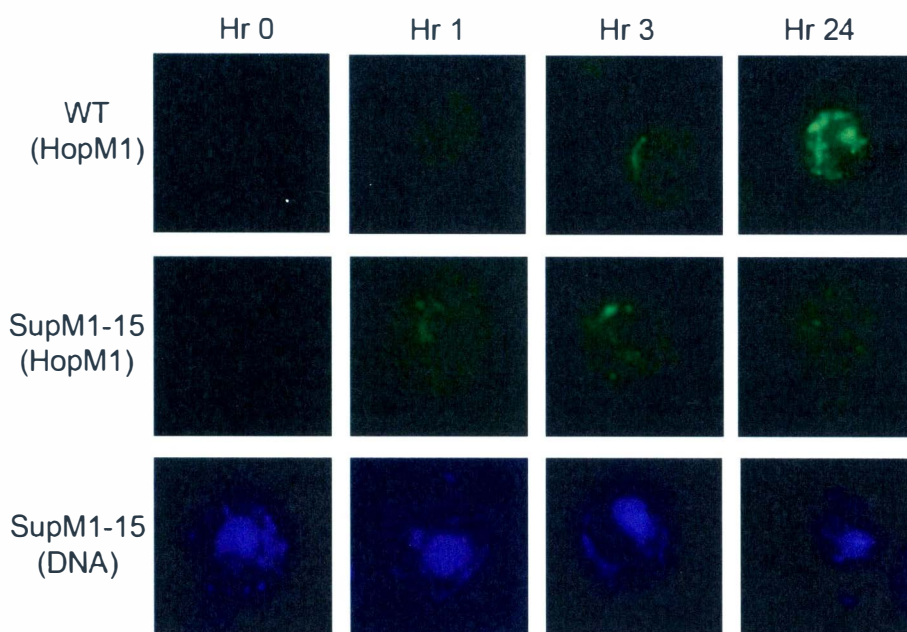


Figure 34. Localization of HopM1 in WT and suppressor yeast strain SupM1-15. Yeast strain JGY4 containing HopM1 (pJB3) and suppressor strain SupM1-15 were grown in selective media containing 2% raffinose. HopM1 was induced after time point zero with the addition of 2% galactose. Aliquots were taken at each time point and fixed with 3.7% formaldehyde for 2 hours. Immunofluorescence was used to visualize the V5 epitope and DAPI to visualize DNA respectively.

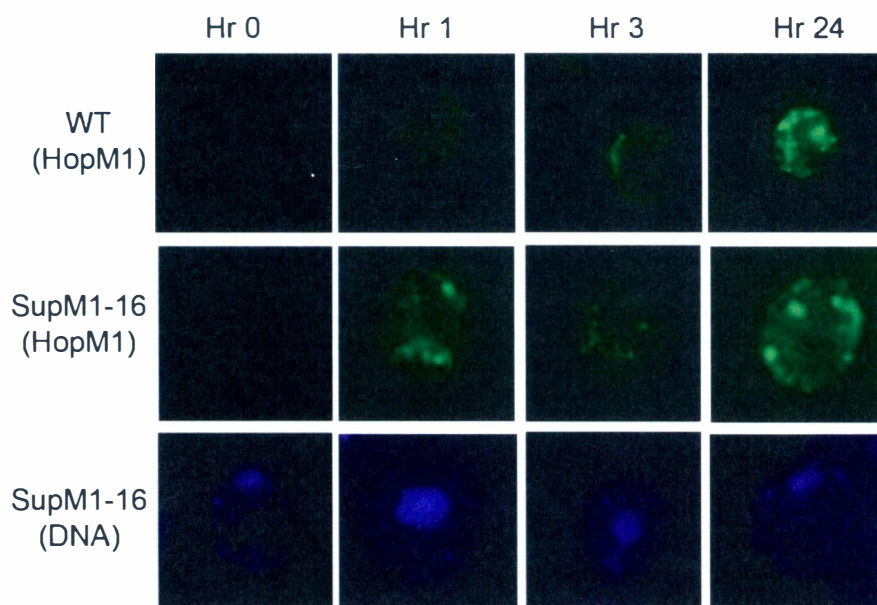


Figure 35. Localization of HopM1 in WT and suppressor yeast strain SupM1-16. Yeast strain JGY4 containing HopM1 (pJB3) and suppressor strain SupM1-16 were grown in selective media containing 2% raffinose. HopM1 was induced after time point zero with the addition of 2% galactose. Aliquots were taken at each time point and fixed with 3.7% formaldehyde for 2 hours. Immunofluorescence was used to visualize the V5 epitope and DAPI to visualize DNA respectively.

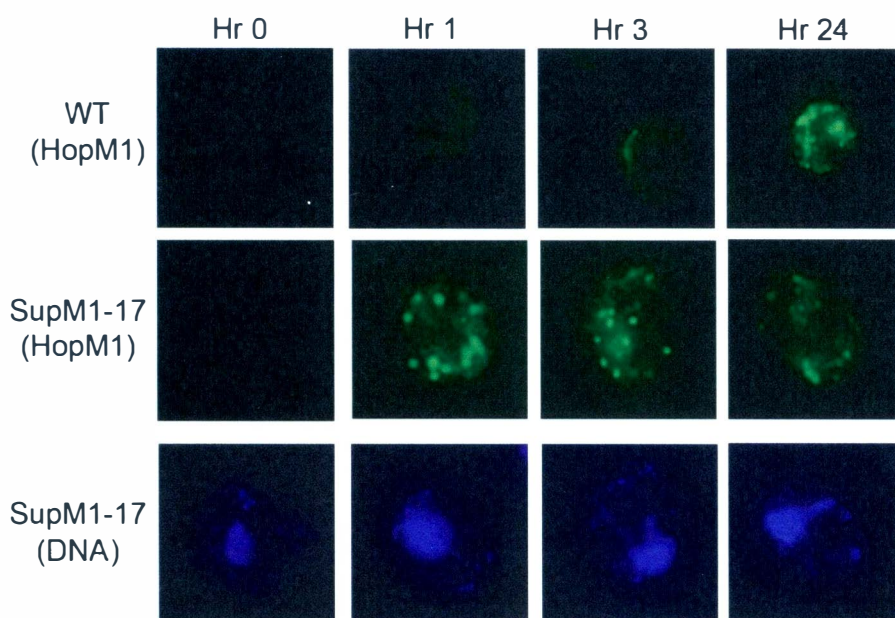


Figure 36. Localization of HopM1 in WT and suppressor yeast strain SupM1-17. Yeast strain JGY4 containing HopM1 (pJB3) and suppressor strain SupM1-17 were grown in selective media containing 2% raffinose. HopM1 was induced after time point zero with the addition of 2% galactose. Aliquots were taken at each time point and fixed with 3.7% formaldehyde for 2 hours. Immunofluorescence was used to visualize the V5 epitope and DAPI to visualize DNA respectively.

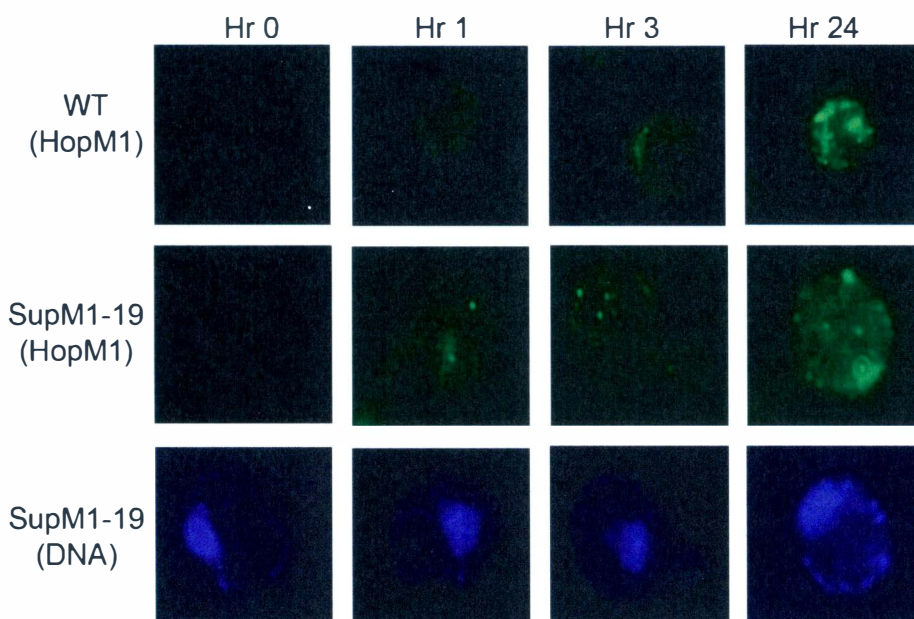


Figure 37. Localization of HopM1 in WT and suppressor yeast strain SupM1-19. Yeast strain JGY4 containing HopM1 (pJB3) and suppressor strain SupM1-19 were grown in selective media containing 2% raffinose. HopM1 was induced after time point zero with the addition of 2% galactose. Aliquots were taken at each time point and fixed with 3.7% formaldehyde for 2 hours. Immunofluorescence was used to visualize the V5 epitope and DAPI to visualize DNA respectively.

Titer Assay of HopM1 Suppressor Strains

In the wild type yeast strain, as described in chapter 2, we observed that HopM1 produced a slow lethal outcome on the yeast cells at 21°C. At hour 24 after induction with galactose, 91% of initial viable cells were remaining and this dropped to 37.5% initial viable cells at hour 48. To determine if there is a change in the trend of the growth

rates of the HopM1 spontaneous suppressor strains, we examined all strains that expressed full length HopM1. We examined the number of cells able to grow on solid dextrose media after being removed from galactose induction. Figures 38-41 show the results for each strain. A majority of the suppressors were able to survive the HopM1 imposed lethality as seen by the increase in the percent of initial viable cells following galactose induction thru hour 6 (for SupM1-2, SupM1-3 and SupM1-14) and thru hour 24 (for SupM1-5 -7, SupM1-9 -10, SupM1-13, SupM1-15 -17 and SupM1-19). One of the suppressors, SupM1-16 (Figure 39), showed a tremendous increase in the percent of initial viable cells from 117.94% at hour 6 to 4205.12% at hour 24.

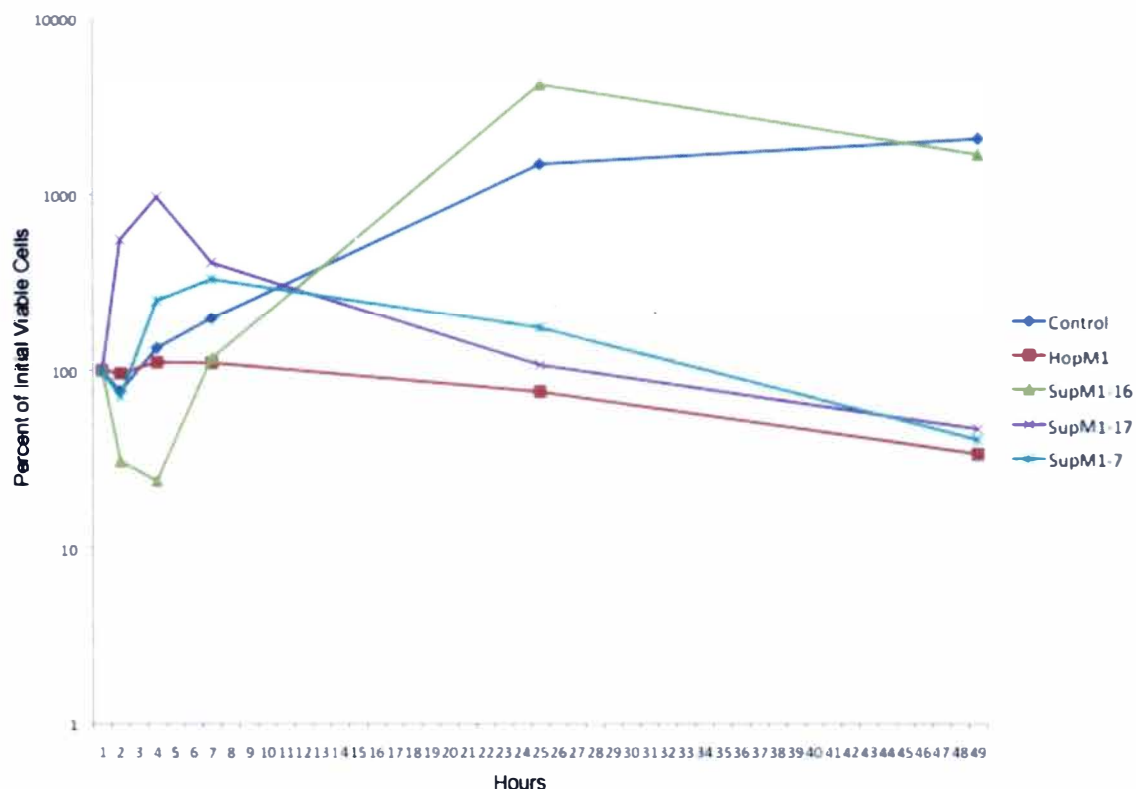


Figure 38. Examination of cell viability of yeast strains containing HopM1. Yeast strain JGY4 containing HopM1 or control plasmid and suppressor strains (SupM1-16, SupM1-17 and SupM1-7) containing HopM1 were grown in selective medium containing 2% raffinose. 2% galactose was added to the medium at the zero time point to induce HopM1 production. 1ml aliquots were taken at each time point, sonicated and serially diluted onto SD-ura-leu plates to identify cells that were able to grow.

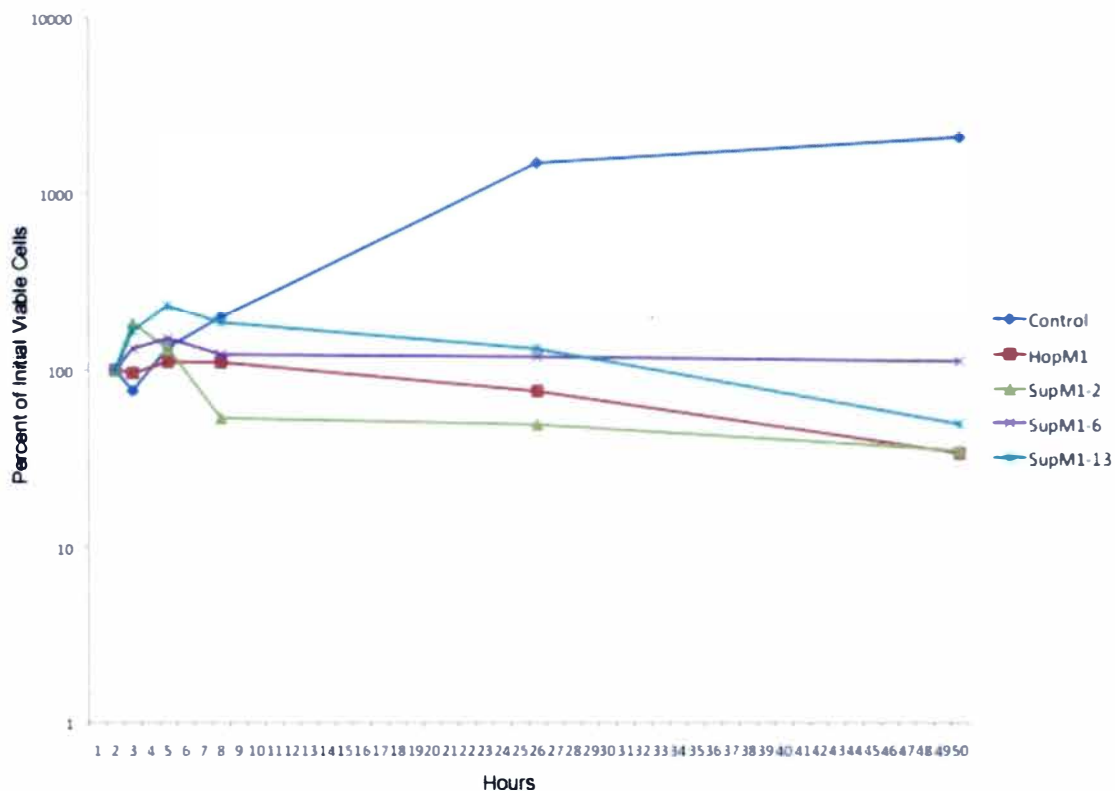


Figure 39. Examination of cell viability of yeast strains containing HopM1. Yeast strain JGY4 containing HopM1 or control plasmid and suppressor strains (SupM1-2, SupM1-6 and SupM1-13) containing HopM1 were grown in selective medium containing 2% raffinose. 2% galactose was added to the medium at the zero time point to induce HopM1 production. 1ml aliquots were taken at each time point, sonicated and serially diluted onto SD-ura-leu plates to identify cells that were able to grow.

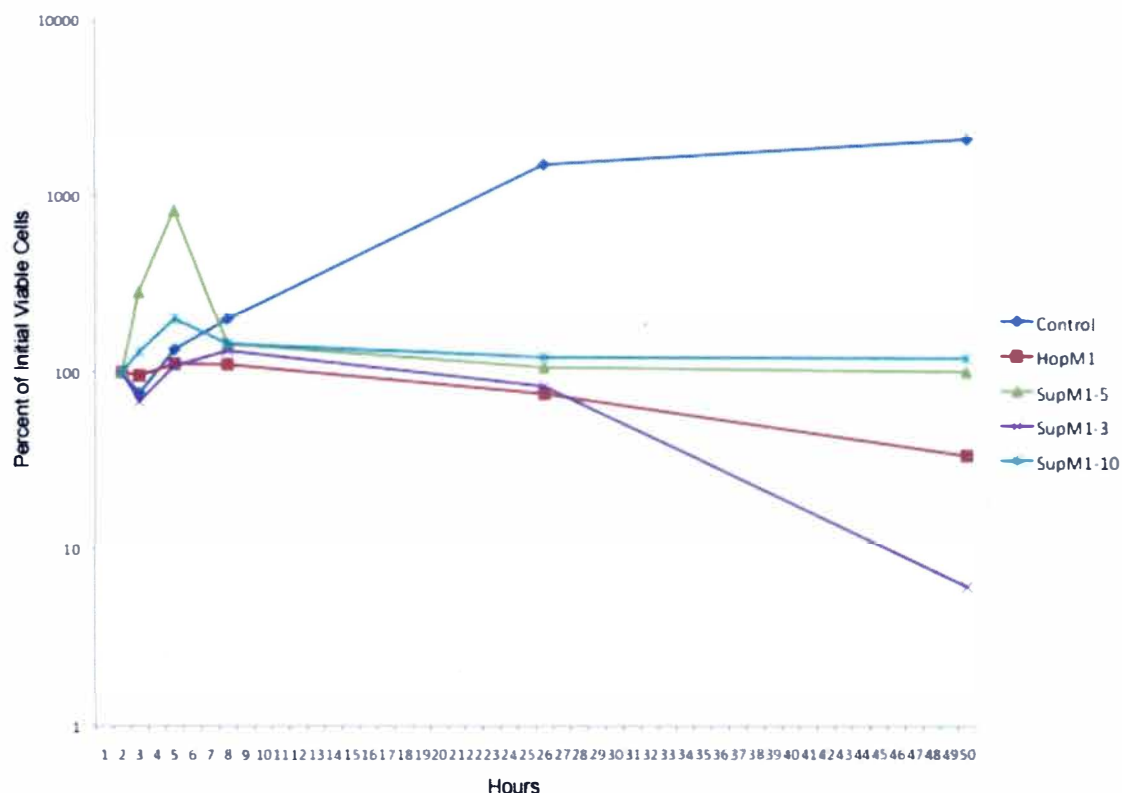


Figure 40. Examination of cell viability of yeast strains containing HopM1. Yeast strain JGY4 containing HopM1 or control plasmid and suppressor strains (SupM1-5, SupM1-3 and SupM1-10) containing HopM1 were grown in selective medium containing 2% raffinose. 2% galactose was added to the medium at the zero time point to induce HopM1 production. 1ml aliquots were taken at each time point, sonicated and serially diluted onto SD-ura-leu plates to identify cells that were able to grow.

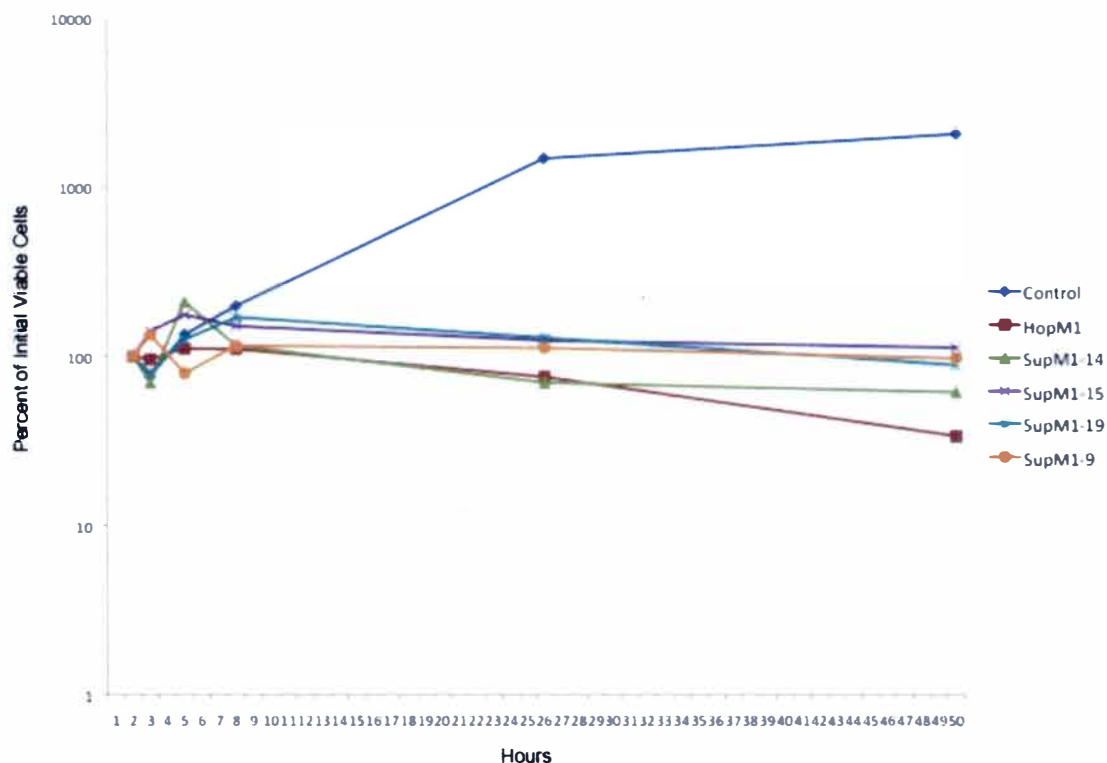


Figure 41. Examination of cell viability of yeast strains containing HopM1. Yeast strain JGY4 containing HopM1 or control plasmid and suppressor strains (SupM1-14, SupM1-15, SupM1-19 and SupM1-9) containing HopM1 were grown in selective medium containing 2% raffinose. 2% galactose was added to the medium at the zero time point to induce HopM1 production. 1ml aliquots were taken at each time point, sonicated and serially diluted onto SD-ura-leu plates to identify cells that were able to grow.

Creation of Genomic Library

To identify the gene(s) responsible for suppression of the HopM1 phenotype in the suppressor strains, we created a genomic library that contained all genes in the suppressor strain. Based on the results from the titer assay, which showed that SupM1-16 showed a significant increase in the percent of initial viable cells as compared to the wild type strain containing HopM1, we decided to clone the gene(s) responsible for growth of that suppressor strain. Genomic DNA was partially digested with *Sau3A* and cloned into the centromeric plasmid vector pRS313. We tried 5 different times using different methods but each time after ligating the partially digested DNA into the vector, we obtained resealed vector that did not integrate the insert. We tried dephosphorylating the ends (removal of 5' phosphate) utilizing shrimp alkaline phosphatase and partially filling the overhang ends of the vector after digesting with *Sau3A* and *Xho1* to prevent the vector from resealing. Each method was 3 times with no result.

Identification of Gene(s) Responsible for Suppression by Whole Genome Sequencing

Lack of success in creating a library led us to consider alternative methods. Current sequencing abilities have decreased the cost that it is no longer cost-

prohibitive to sequence the entire genome of yeast. We contracted with Otogenetics to sequence our wild type strain as a control and one suppressor strain, SupM1-16.

Genomic DNA for yeast strain JGY4 and SupM1-16 were prepared and sent off for sequencing. *Saccharomyces cerevisiae* has 12,071,326 bases. 475,207,000 bases were sequenced for strain JGY4, with an average of 100bp per read and 40 repeats per read. 79.5% were mapped reads and the remaining 20.5% were attributed to repetitive sequences, ribosomal RNA, primers and low quality reads. 583,978,000 bases were sequenced for strain SupM1-16. The average base length that was read was 100bp with 40 repeats per read. For strain SupM1-16, there was 81.93% mapped reads. The remaining 18.07% non-mapped reads were from repetitive sequences, ribosomal RNA, primers and low quality reads.

Analysis of Sequence Data

Examining the sequence data, we saw that the WT strain had 5555 base changes and data from SupM1-16 showed 5391 base changes. These changes were based on comparing the each strain against the original reference sequence of *Saccharomyces cerevisiae*. Our strain is related, but has likely diverged since it has been passed from lab to lab

for the past 20 years or so. We filtered thru the data and compared the sequence data of SupM1-16 against the sequence of the WT strain, as this is the background strain our suppressors were created in. We discarded changes in bases that were the same in the wild type and SupM1-16, after which this left us with 146 possible mutations found in SupM1-16. Table 6 shows a summary of the results obtained for the 2 strains. After examining the sequence data, and based on our knowledge that we have to account for common sequence errors, misalignment from computer algorithm and the likelihood that a given change would result in a particular mutation we found 8 changes (6 genes) that could be responsible for the suppression seen in the mutant (Table 7). Based on the size of the yeast genome and the mutation rate of 10^7 , the number of mutations we obtained is relatively close to the number that is expected.

Strain	Initial Change	Filtered	Potential Genes
WT	5555	0	0
SupM1-16	5391	146	6

Table 6. The two yeast genomes that were sequenced.

The simplest scenario is that only one of the 6 identified genes with a mutation is the gene responsible for suppression of HopM1 in the SupM1-16 strain. To determine which gene is responsible, we prioritized the genes based on what we know about HopM1 and its location in the cell. *RPS25A* is nearly homologous to *RPS25B* and as such not likely responsible (Planta and Mager, 1998). *YGR064W* is a dubious open reading frame that does not likely encode a protein. Furthermore it overlaps *SPT4* on the opposite strand. *SPT4* and *NET1* are nuclear proteins (Rondon et al., 2004, Straight et al., 1999) and since we see no evidence of localization to nucleus, these were least likely to be responsible. *tT(UGU)Q1* is a tRNA in mitochondria, and since there is no HopM1 effect in the mitochondria, this is not likely as well (Foury et al., 1998). This leaves us with *RSP5* remaining as a likely candidate for SupM1-16.

Potential Gene	Identity	Genetic change	Change that occurred
<i>RSP5</i>	E3 Ubiquitin Ligase	G→T	Gly → Val
<i>SPT4</i>	Suppressor of Ty's	A→C	Met → Arg
<i>YGR064W</i>	Dubious open reading frame	A→C	His → Pro
<i>NET1</i>	Nucleolar silencing establishing factor and telophase regulator	C→T	Ser → Leu
<i>RPS25A</i>	Ribosomal Protein of the Small Subunit	A→G	UTR
<i>tT(UGU)Q1</i>	Mitochondrial Threonine tRNA	Deletion	UTR

Table 7. The potential genes identified from sequencing and data analysis of SupM1-16.

RSP5 is an E3 Ubiquitin Ligase that is involved in regulating many cellular processes such as multivesicular body sorting, heat shock response, ubiquitination, and endocytosis (Wang et al., 1995). Nomura et al., 2006, have suggested that HopM1 acts as an adaptor that targets *Arabidopsis thaliana* AtMIN7 (a guanine nucleotide exchange factor) to the proteasome for degradation. Our results (chapter 2) show that HopM1 localizes to organelles or vesicles of the secretory pathway. Thus we have continued with *RSP5* as a likely candidate and will reassess as we proceed.

We utilized PCR to amplify *RSP5* from genomic DNA that was obtained from SupM1-16 using the forward and reverse primer listed in table 8. We engineered a *SacI* restriction enzyme site onto our reverse primer to allow for ease of cloning into the yeast vector pRS313. Our forward primer contained a *HindIII* restriction enzyme site. Once the gene was amplified and verified, it was ligated into the yeast vector and transformed into DH5 α cells. Clones were processed and sequenced to verify that they contained the Glycine to Valine mutation that was determined from sequencing of the suppressor genome, and did not contain any other mutation. Table 8 shows the oligonucleotides used for PCR amplification and sequence verification.

The correct clones were transformed into the yeast strain expressing 2 HopM1 plasmids to look for suppression. Results at present are inconclusive. *RSP5* does not seem to be suppressing the effects of HopM1, however there are a number of reasons behind this. One reason is the presence of the WT copy of *RSP5* in our yeast strain. One way to overcome this would be to over express the mutant copy of *RSP5* and look for suppression. If we see suppression, we would have successfully identified the suppressor gene. If *RSP5* does not suppress the effects of HopM1, we will return to the other 5 potential genes (listed in table 7) and pick another one to clone and repeat the process until we identify the genetic change that is responsible for suppression.

Oligonucleotide	Sequence
RSP5-F1	AGTTTGAGGAAAGCTTGGGAGAG
RSP5-R1	GACGACGAGCTCGTAGGTGACTGGCAAGAGAG
RSP5-1	CGATCATGTGATCTTACGTAC
RSP5-2	GAAGCACATTTTGCAAGTGA
RSP5-3	CCGTTCCCCGGATCCGT
RSP5-4	CGTCAAGTCCGCATTAC
RSP5-5	CCAACGCTCGATCAAACAG
RSP5-6	CCGGTCTCCCAATTAGGT
RSP5-7	CGGTGGTGTTTCCAGAG
RSP5-8	GGTGAAGTTGTAACAGTGGA
RSP5-9	CTTCTCGTATACCTGTCAAC
RSP5-10	AGCAACATGGAAAGCAGC

Table 8. List of oligonucleotides used for sequence verification.

Discussion

The suppressor assay developed was successful in producing spontaneous suppressors of HopM1 imposed lethality. We were able to identify 19 haploid spontaneous suppressor strains that were capable of surviving the HopM1 imposed lethality. Of the 19, three of the suppressors, SupM1-1, SupM1-11 and SupM1-18 were not able to sporulate. These suppressors were not considered further.

Characterization of the suppressors has focused on determining: stability of HopM1 in each suppressor strain and localization of HopM1 in the suppressor strains. On examining our results, we discovered that five of the suppressors (SupM1-1, SupM1-4, SupM1-8, SupM1-11 and SupM1-12) produced degradation products of the HopM1 protein. While this may be telling us something important about the mechanism, we decided to focus on the remaining 13 (SupM1-2, SupM1-3, SupM1-5, SupM1-6, SupM1-7, SupM1-9, SupM1-10, SupM1-13, SupM1-14, SupM1-15, SupM1-16, SupM1-17, and SupM1-19), which produced full length HopM1 (75kDa) with no degradation. We chose to discontinue analysis of suppressors SupM1-1, SupM1-4, SupM1-8, SupM1-11 and SupM1-12 from further characterization, as we were concerned that these suppressors were able to survive due to not having

full length HopM1 present and showed a similar degradation pattern of HopM1 at 30°C and 37°C. The rationale behind discarding these suppressors is due to the probability of these suppressors functioning as proteases and therefore degrading the HopM1 protein, thus allowing the yeast to survive the HopM1 imposed lethality because full length HopM1 is no longer present.

Examination of HopM1 localization in the remaining 13 suppressors showed staining in punctate regions in the cytoplasm. We presume these are secretory organelles as described in an earlier chapter (see chapter 2). From looking at each of the suppressors, we found no difference in localization of HopM1 in the suppressor strains as compared to wild type. We began to see localization of HopM1 to punctate like structures beginning at hour 1 thru hour 24, with no obvious localization to the nucleus.

We utilized the titer assay in order to determine if there was a change in growth rates of the suppressor strains containing HopM1 plasmids compared to wild type containing HopM1 plasmids. We observed that one of the suppressors, SupM1-16, showed a significant difference in growth rate. At hour 6 there are about 117.94% of initial viable cells present and at hour 24, this number increases to 4205.12% as compared to 110.56% and 75.60% present at

the respective time points for HopM1 in the wild type strain. Due to the difference in growth and ability to survive the HopM1 lethality, we picked this suppressor strain as a candidate to move forward with identification of the suppressor gene or genes.

The entire genome of SupM1-16 was sequenced and compared to the wild type background (JGY4) that the suppressors were created in. On comparison, we identified 6 genes that could be responsible for suppression. We selected the gene *RSP5* to begin the identification process. *RSP5* was selected based on a multitude of factors. Firstly, research done by Nomura et al, 2006 in *Arabidopsis thaliana* indicates that HopM1 serves as a possible adaptor for AtMIN7, a guanine nucleotide exchange factor and targets it to the 26S proteasome (Nomura et al, 2006, Pickart, 2001) for degradation.

On looking at the sequence of *RSP5* and comparing it to the published sequence, we observed a single base pair mutation in the HECT domain at position 753 that causes a change from a Glycine to a Valine. Both amino acids are non-polar amino acids with the exception of the side chain of Valine being slightly longer than that of Glycine. This change in amino acid could possibly change the way

ubiquitin forms the thioester bond with the conserved Cysteine that is 24 amino acids away from the mutation.

RSP5 is an E3 ubiquitin ligase that is involved in regulating many cellular processes such as multivesicular body sorting, heat shock response, ubiquitination of substrates and shuttling them to the proteasome, and endocytosis (Wang et al., 1999, Pickart, 2001, Shirssekar et al., 2010). A literature search indicated that *SEC7*, *GEA1* and *GEA2* are the three yeast homologs of guanine nucleotide exchange factors (GNEF) for ADP ribosylation factors that are involved in endocytosis (Achstetter et al., 1988). We hypothesize that either one of these homologs binds to HopM1 and changes the conformation thus preventing ubiquitin from interacting with *RSP5* and disrupts the degradation process allowing vesicle formation to occur normally. This disruption could possibly be caused by either the mutation in the HECT domain of *RSP5* therefore preventing ubiquitin from being transferred to the conserved Cysteine site or by no longer being able to accept ubiquitin from the E2 site. This fact also ties in our discovery from an earlier chapter that HopM1 localizes to the mitochondria and organelles of the secretory pathway.

Future work that could stem from this would be utilizing the yeast two-hybrid system to determine whether RSP5, HopM1 and other components like *SEC7*, *GEA1* or *GEA2* *physically interact*. Other possible work would be to perform co-immunoprecipitation to determine if we can identify any interaction between HopM1 and/or RSP5 with *SEC7*, *GEA1* or *GEA2* and therefore support our hypothesis that HopM1 binds to either of these cellular targets.

CHAPTER 4

CONCLUSION

Work on this project began with very little information available on HopM1, an effector protein of *Pseudomonas syringae* pv. *tomato* DC3000. Throughout the preceding chapters, I have been able to show that HopM1 has an effect and plays a significant role. With this work, using *Saccharomyces cerevisiae* as a model system, I have been able to show that HopM1 shows a lethal phenotype at 21°C and localizes to the organelles or vesicles of the secretory pathway. Munkvold et al., 2008, published research indicating that HopM1 does not have an effect in yeast at 30°C. The discrepancy in the findings stem from the fact that their work was done at 30°C and ours was done at 21°C. Another fact is that literature also indicates that the effector proteins from *Pseudomonas syringae* pv. *tomato* DC3000 do not elicit an effect in tomato plants at temperatures higher than 25°C but instead prefer cool and moist climates to be able to elicit an effect (Preston, 2000).

Localization studies of HopM1, the identification of spontaneous suppressors and the identification of the

gene(s) responsible for the suppression of the HopM1 lethal phenotype have lead to the discovery that HopM1 plays a possible role in endocytosis and the secretory pathway. Preliminary results indicate that *RSP5* may be the gene responsible for suppression. There is work still left to be done to confirm this result, but we are confident that this is true based on the findings as indicated by Nomura et al. Work done by Nomura et al., 2006 has helped shed some light on the function of HopM1. Their work showed that HopM1 acts as a possible adaptor that targets the guanine nucleotide exchange factor and shuttles it to the proteasome for degradation.

Future work that could stem from this work would be genome sequencing of the remaining spontaneous suppressors to identify other gene responsible for suppression and the long term goal of possibly identifying a genetic or biochemical pathway that is involved. All of this is needed to help shed some light on the function of HopM1 and its role in the cell.

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